# Fig. 1

## 2H7scFv-Ig cDNA and predicted amino acid sequence:

```
        HindIII      NcoI              2H7 VL Leader Peptide→
        ~~~~~~       ~~~~~~

                     M   D   F   Q   V   Q   I   F   S   F   L   L   I   S   A   S
    1   AAGCTTGCCG CCATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA


                                       2H7 VL→
        V   I   I   A   R   G   Q   I   V   L   S   Q   S   P   A   I   L   S   A   S
   61   GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT


        P   G   E   K   V   T   M   T   C   R   A   S   S   S   V   S   Y   M   H   W
  121   CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG


                            BamHI
                            ~~~~~~~
        Y   Q   Q   K   P   G   S   S   P   K   P   W   I   Y   A   P   S   N   L   A
  181   TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT


        S   G   V   P   A   R   F   S   G   S   G   S   G   T   S   Y   S   L   T   I
  241   TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC


        S   R   V   E   A   E   D   A   A   T   Y   Y   C   Q   Q   W   S   F   N   P
  301   AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA


        (Gly4Ser)3 Linker
        P   T   F   G   A   G   T   K   L   E   L   K   G   G   G   S   G   G   G
  361   CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT


                                                              2H7 VH →
        G   S   G   G   G   S   S   Q   A   Y   L   Q   Q   S   G   A   E   L   V
  421   GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG


        R   P   G   A   S   V   K   M   S   C   K   A   S   G   Y   T   F   T   S   Y
  481   AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC


        N   M   H   W   V   K   Q   T   P   R   Q   G   L   E   W   I   G   A   I   Y
  541   AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT


        P   G   N   G   D   T   S   Y   N   Q   K   F   K   G   K   A   T   L   T   V
  601   CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA


        D   K   S   S   S   T   A   Y   M   Q   L   S   S   L   T   S   E   D   S   A
  661   GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG


        V   Y   F   C   A   R   V   V   Y   Y   S   N   S   Y   W   Y   F   D   V   W
  721   GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

# Fig. 1 (continued)

```
                              BclI
                   ~~~~~~~human IgG1 Fc domain →
         G   T   G   T   T   V   T   V   S   D   Q   E   P   K   S   C   D   K   T   H
   781   GGCACAGGGA CCACGGTCAC CGTCTCTGAT CAGGAGCCCA AATCTTGTGA CAAAACTCAC


         T   C   P   P   C   P   A   P   E   L   L   G   G   P   S   V   F   L   F   P
   841   ACATGCCCAC CGTGCCCAGC ACCTGAACTC CTGGGGGGAC CGTCAGTCTT CCTCTTCCCC


         P   K   P   K   D   T   L   M   I   S   R   T   P   E   V   T   C   V   V   V
   901   CCAAAACCCA AGGACACCCT CATGATCTCC CGGACCCCTG AGGTCACATG CGTGGTGGTG


         D   V   S   H   E   D   P   E   V   K   F   N   W   Y   V   D   G   V   E   V
   961   GACGTGAGCC ACGAAGACCC TGAGGTCAAG TTCAACTGGT ACGTGGACGG CGTGGAGGTG


         H   N   A   K   T   K   P   R   E   E   Q   Y   N   S   T   Y   R   V   V   S
  1021   CATAATGCCA AGACAAAGCC GCGGGAGGAG CAGTACAACA GCACGTACCG TGTGGTCAGC


         V   L   T   V   L   H   Q   D   W   L   N   G   K   E   Y   K   C   K   V   S
  1081   GTCCTCACCG TCCTGCACCA GGACTGGCTG AATGGCAAGG AGTACAAGTG CAAGGTCTCC


         N   K   A   L   P   A   P   I   E   K   T   I   S   K   A   K   G   Q   P   R
  1141   AACAAAGCCC TCCCAGCCCC CATCGAGAAA ACAATCTCCA AGCCAAAGG GCAGCCCCGA


         E   P   Q   V   Y   T   L   P   P   S   R   D   E   L   T   K   N   Q   V   S
  1201   GAACCACAGG TGTACACCCT GCCCCCATCC CGGGATGAGC TGACCAAGAA CCAGGTCAGC


         L   T   C   L   V   K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N
  1261   CTGACCTGCC TGGTCAAAGG CTTCTATCCC AGCGACATCG CCGTGGAGTG GGAGAGCAAT


         G   Q   P   E   N   N   Y   K   T   T   P   P   V   L   D   S   D   G   S   F
  1321   GGGCAGCCGG AGAACAACTA CAAGACCACG CCTCCCGTGC TGGACTCCGA CGGCTCCTTC


         F   L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G   N   V   F   S
  1381   TTCCTCTACA GCAAGCTCAC CGTGGACAAG AGCAGGTGGC AGCAGGGGAA CGTCTTCTCA


         C   S   V   M   H   E   A   L   H   N   H   Y   T   Q   K   S   L   S   L   S
  1441   TGCTCCGTGA TGCATGAGGC TCTGCACAAC CACTACACGC AGAAGAGCCT CTCCCTGTCT


                          XbaI
                          ~~~~~
         P   G   K   *   S   R
  1501   CCGGGTAAAT GATCTAGA
```
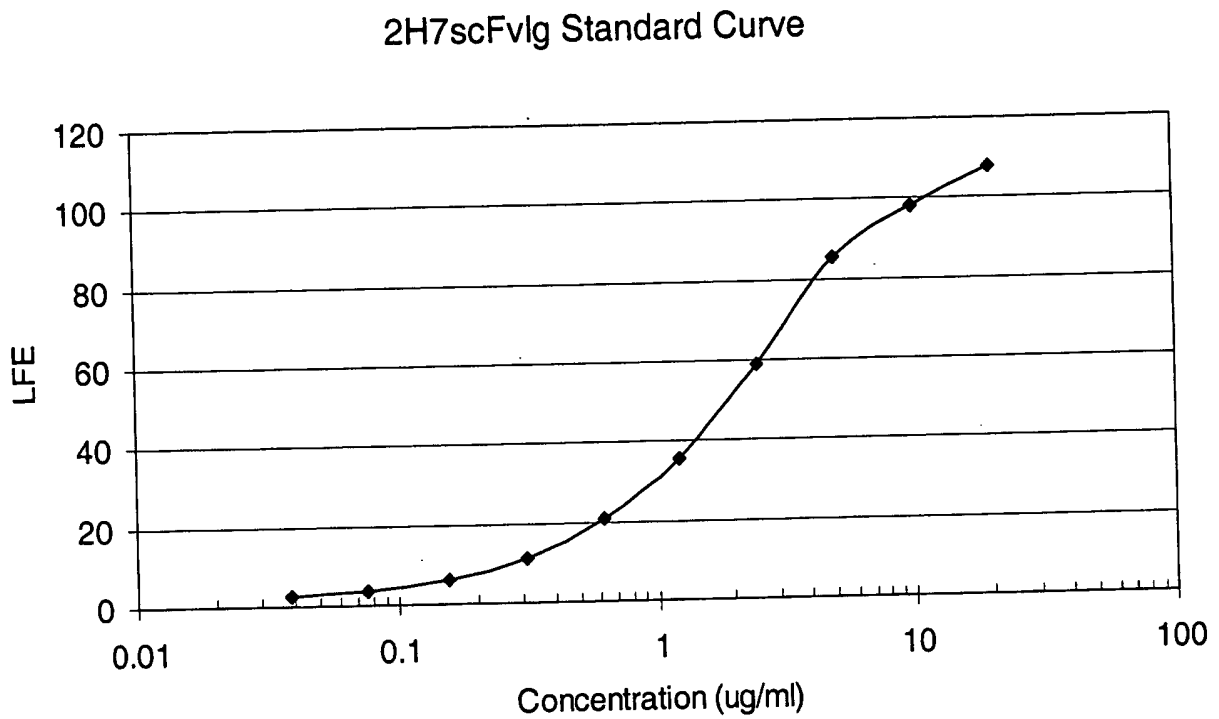
# Fig. 2
## Production Levels of 2H7 scFvIgG1 (SSS-S)H WCH2 WCH3 by Stable CHO Lines

### 2H7scFvIg Standard Curve



| Clone | LFE @ 1:50 Estimated Concentration (µg/ml) |
|---|---|
| D2 | 26.156 |
| IIIC6 | 25.755 |
| IVA3 | 28.661 |
| Spent bulk | 29.664 |

# Fig. 3
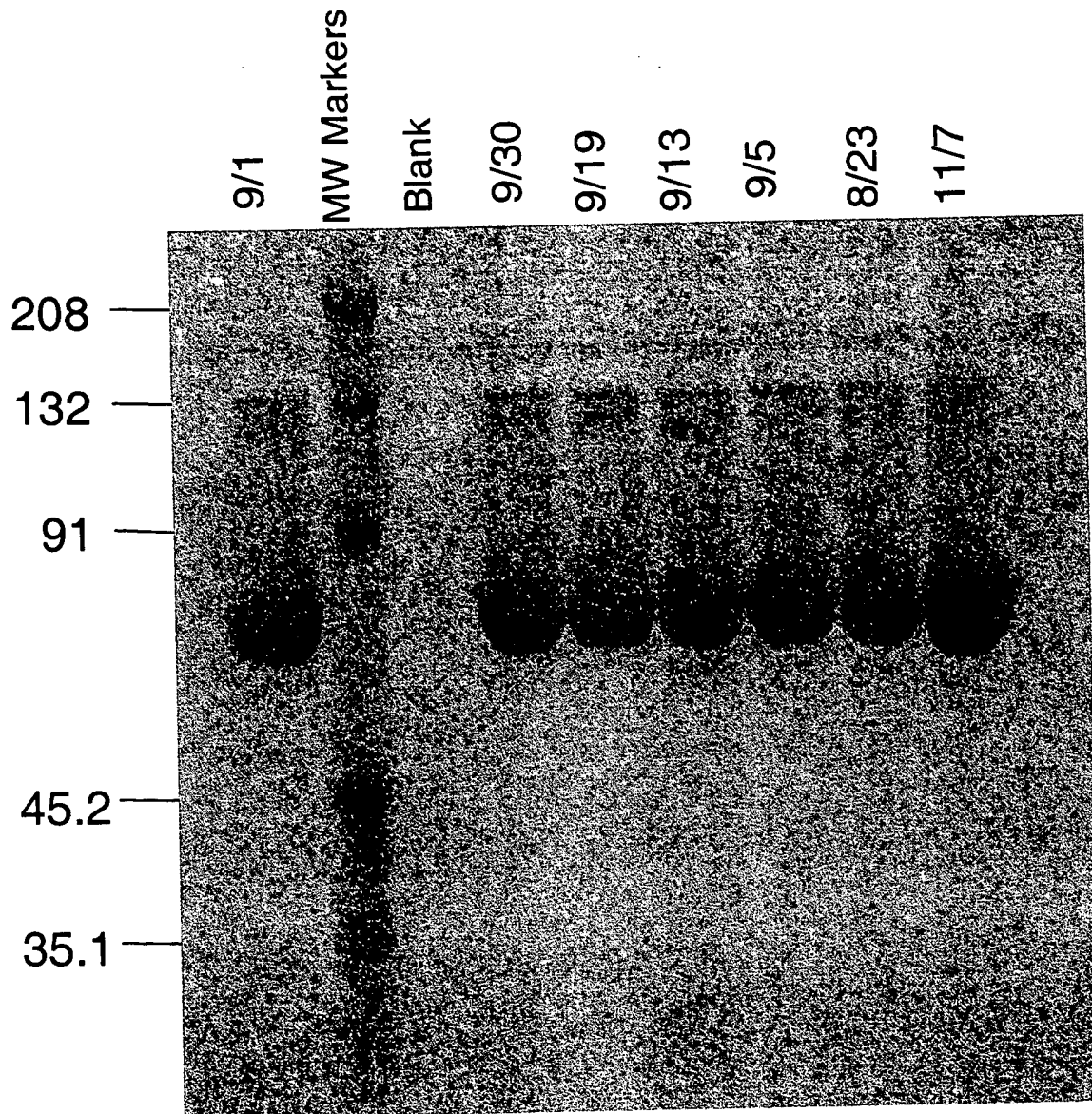SDS-PAGE Analysis of
2H7 scFvIgG1 (SSS-S)H WCH2 WCH3 Protein.

# Fig. 4A

### Complement Mediated B Cell Killing After Binding of
### CD20-targeted 2H7 scFvIgG1 (SSS-S)H WCH2 WCH3:

| 2H7scFv-Ig Concentration | RAMOS # live cells/total cells | | BJAB # live cells/total cells | |
|---|---|---|---|---|
| 20 µg/ml + complement | - | 0.16 | - | 0.07 |
| 5 µg/ml + complement | - | 0.2 | - | N.D. |
| 1.25 µg/ml + complement | - | 0.32 | - | 0.1 |
| Complement alone | - | 0.98 | - | 0.94 |

*Viability was determined by trypan blue exclusion and is tabulated as the fraction of viable cells out of the total number of cells counted.

**N.D. (not determined).

# Fig. 4B

Antibody-dependent cellular cytotoxicity (ADCC) mediated by 2H7scFv-IgG1 (SSS-S)H WCH2 WCH3:
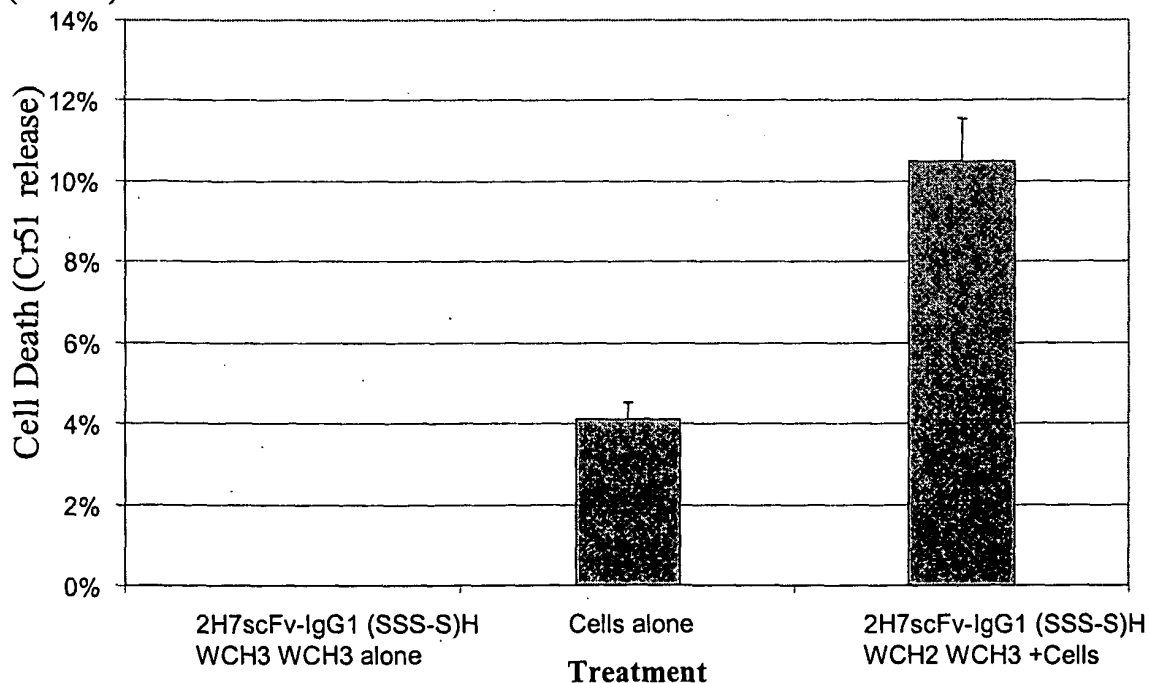
# Fig. 5

Effects of Crosslinking of CD20 and CD40 Cell Surface Receptors

on B Cell Proliferation:



CPM INCORPORATED
(counts per minute)
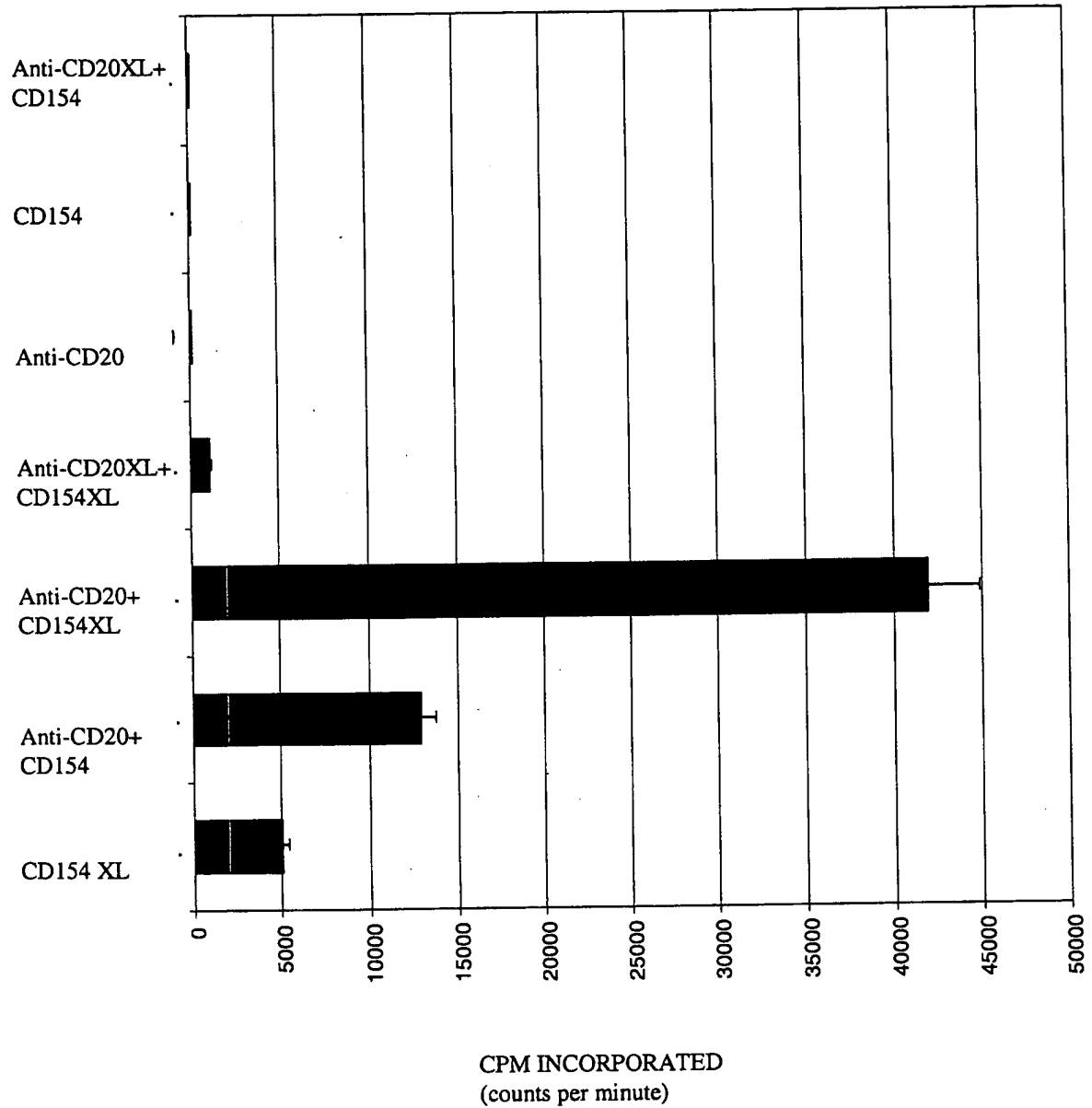
# Fig. 6

## Effect of Simultaneous ligation of CD20 and CD40 on CD95 and apoptosis.

**Fig. 6A.**



**Fig 6B.**



Stimuli

# Fig. 7A

## 2H7-CD154 L2 cDNA and predicted amino acid sequence:

```
HindIII       NcoI    2H7 V_L Leader Peptide →
~~~~~~        ~~~~~~

                      M   D   F   Q   V   Q   I   F   S   F   L   L   I   S   A   S
      1    AAGCTTGCCG CC    ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA


                              2H7 V_L →
           V   I   I   A   R   G   Q   I   V   L   S   Q   S   P   A   I   L   S   A   S
     61    GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT


           P   G   E   K   V   T   M   T   C   R   A   S   S   S   V   S   Y   M   H   W
    121    CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG


                  BamHI
                  ~~~~~~~
           Y   Q   Q   K   P   G   S   S   P   K   P   W   I   Y   A   P   S   N   L   A
    181    TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT


           S   G   V   P   A   R   F   S   G   S   G   S   G   T   S   Y   S   L   T   I
    241    TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC


           S   R   V   E   A   E   D   A   A   T   Y   Y   C   Q   Q   W   S   F   N   P
    301    AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA


    (Gly_4 Ser)_3 Linker →
           P   T   F   G   A   G   T   K   L   E   L   K   G   G   G   S   G   G   G
    361    CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT


                                              2H7 V_H →
           G   S   G   G   G   S   S   Q   A   Y   L   Q   Q   S   G   A   E   L   V
    421    GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG


           R   P   G   A   S   V   K   M   S   C   K   A   S   G   Y   T   F   T   S   Y
    481    AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG CTACACATT TACCAGTTAC


           N   M   H   W   V   K   Q   T   P   R   Q   G   L   E   W   I   G   A   I   Y
    541    AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT


           P   G   N   G   D   T   S   Y   N   Q   K   F   K   G   K   A   T   L   T   V
    601    CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA


           D   K   S   S   S   T   A   Y   M   Q   L   S   S   L   T   S   E   D   S   A
    661    GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG


           V   Y   F   C   A   R   V   V   Y   Y   S   N   S   Y   W   Y   F   D   V   W
    721    GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

## Fig. 7A (continued)

### human CD154/amino acid 48→

```
                                        Bcl/Bam hybrid site
        G   T   G   T    T   V   T    V   S   D    P   R   R   L    D   K   I    E   D   E
  781   GGCACAGGGA CCACGGTCAC CGTCTCTGAT CCAAGAAGGT TGGACAAGAT AGAAGATGAA

        R   N   L   H    E   D   F    V   F   M    K   T   I    Q   R   C   N    T   G   E
  841   AGGAATCTTC ATGAAGATTT TGTATTCATG AAAACGATAC AGAGATGCAA CACAGGAGAA

        R   S   L   S    L   L   N    C   E   E    I   K   S   Q    F   E   G    F   V   K
  901   AGATCCTTAT CCTTACTGAA CTGTGAGGAG ATTAAAAGCC AGTTTGAAGG CTTTGTGAAG

                                                                          BclI
        D   I   M   L    N   K   E    E   T   K    K   E   N   S    F   E   M    Q   K   G
  961   GATATAATGT TAAACAAAGA GGAGACGAAG AAAGAAAACA GCTTTGAAAT GCAAAAAGGT

  BclI
  ~~~~~
        D   Q   N   P    Q   I   A    A   H   V    I   S   E   A    S   S   K    T   T   S
 1021   GATCAGAATC CTCAAATTGC GGCACATGTC ATAAGTGAGG CCAGCAGTAA AACAACATCT

        V   L   Q   W    A   E   K    G   Y   Y    T   M   S   N    N   L   V    T   L   E
 1081   GTGTTACAGT GGGCTGAAAA AGGATACTAC ACCATGAGCA ACAACTTGGT AACCCTGGAA

        N   G   K   Q    L   T   V    K   R   Q    G   L   Y   Y    I   Y   A    Q   V   T
 1141   AATGGGAAAC AGCTGACCGT TAAAAGACAA GGACTCTATT ATATCTATGC CCAAGTCACC

                         HindIII
                         ~~~~~~~
        F   C   S   N    R   E   A    S   S   Q    A   P   F   I    A   S   L    C   L   K
 1201   TTCTGTTCCA ATCGGGAAGC TTCGAGTCAA GCTCCATTTA TAGCCAGCCT CTGCCTAAAG

        S   P   G   R    F   E   R    I   L   L    R   A   A   N    T   H   S    S   A   K
 1261   TCCCCCGGTA GATTCGAGAG AATCTTACTC AGAGCTGCAA ATACCCACAG TTCCGCCAAA

        P   C   G   Q    Q   S   I    H   L   G    G   V   F   E    L   Q   P    G   A   S
 1321   CCTTGCGGGC AACAATCCAT TCACTTGGGA GGAGTATTTG AATTGCAACC AGGTGCTTCG

                                                         NcoI
                                                         ~~~~~~~
        V   F   V   N    V   T   D    P   S   Q    V   S   H   G    T   G   F    T   S   F
 1381   GTGTTTGTCA ATGTGACTGA TCCAAGCCAA GTGAGCCATG GCACTGGCTT CACGTCCTTT

                         XhoI            XbaI
                         ~~~~~~          ~~~~
        G   L   L   K    L   E   *    *   S   R
 1441   GGCTTACTCA AACTCGAGTG ATAATCTAGA
```

# Fig. 7B.

## 2H7scFv-CD154 S4 cDNA and predicted amino acid sequence:

```
HindIII      NcoI
~~~~~~      ~~~~~~2H7 V_L Leader Peptide→
             M   D   F   Q   V   Q   I   F   S   F   L   L   I   S   A   S
1    AAGCTTGCCG CC   ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                                2H7 V_L →
     V   I   I   A   R   G   Q   I   V   L   S   Q   S   P   A   I   L   S   A   S
61   GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

     P   G   E   K   V   T   M   T   C   R   A   S   S   S   V   S   Y   M   H   W
121  CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                     BamHI
                     ~~~~~~~
     Y   Q   Q   K   P   G   S   S   P   K   P   W   I   Y   A   P   S   N   L   A
181  TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

     S   G   V   P   A   R   F   S   G   S   G   S   G   T   S   Y   S   L   T   I
241  TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

     S   R   V   E   A   E   D   A   A   T   Y   Y   C   Q   Q   W   S   F   N   P
301  AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

(Gly_4Ser)_3 Linker →
     P   T   F   G   A   G   T   K   L   E   L   K   G   G   G   S   G   G   G
361  CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                                          2H7 V_H →
     G   S   G   G   G   S   S   Q   A   Y   L   Q   Q   S   G   A   E   L   V
421  GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

     R   P   G   A   S   V   K   M   S   C   K   A   S   G   Y   T   F   T   S   Y
481  AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

     N   M   H   W   V   K   Q   T   P   R   Q   G   L   E   W   I   G   A   I   Y
541  AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

     P   G   N   G   D   T   S   Y   N   Q   K   F   K   G   K   A   T   L   T   V
601  CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

     D   K   S   S   S   T   A   Y   M   Q   L   S   S   L   T   S   E   D   S   A
661  GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

     V   Y   F   C   A   R   V   V   Y   Y   S   N   S   Y   W   Y   F   D   V   W
721  GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

# Fig. 7B

human CD154/amino acid 108 →

```
                           Bcl/Bam hybrid site
     BclI
        G   T   G   T   T   V   T   V   S   D   P   E   N   S   F   E   M   Q   K   G
781     GGCACAGGGA CCACGGTCAC CGTCTCTGAT CCAGAAAACA GCTTTGAAAT GCAAAAAGGT

     BclI
     ~~~~~
        D   Q   N   P   Q   I   A   A   H   V   I   S   E   A   S   S   K   T   T   S
841     GATCAGAATC CTCAAATTGC GGCACATGTC ATAAGTGAGG CCAGCAGTAA AACAACATCT

        V   L   Q   W   A   E   K   G   Y   Y   T   M   S   N   N   L   V   T   L   E
901     GTGTTACAGT GGGCTGAAAA AGGATACTAC ACCATGAGCA ACAACTTGGT AACCCTGGAA

        N   G   K   Q   L   T   V   K   R   Q   G   L   Y   Y   I   Y   A   Q   V   T
961     AATGGGAAAC AGCTGACCGT TAAAAGACAA GGACTCTATT ATATCTATGC CCAAGTCACC

                           HindIII
                           ~~~~~~~
        F   C   S   N   R   E   A   S   S   Q   A   P   F   I   A   S   L   C   L   K
1021    TTCTGTTCCA ATCGGGAAGC TTCGAGTCAA GCTCCATTTA TAGCCAGCCT CTGCCTAAAG

        S   P   G   R   F   E   R   I   L   L   R   A   A   N   T   H   S   S   A   K
1081    TCCCCCGGTA GATTCGAGAG AATCTTACTC AGAGCTGCAA ATACCCACAG TTCCGCCAAA

        P   C   G   Q   Q   S   I   H   L   G   G   V   F   E   L   Q   P   G   A   S
1141    CCTTGCGGGC AACAATCCAT TCACTTGGGA GGAGTATTTG AATTGCAACC AGGTGCTTCG

                                            NcoI
                                            ~~~~~~~
        V   F   V   N   V   T   D   P   S   Q   V   S   H   G   T   G   F   T   S   F
1201    GTGTTTGTCA ATGTGACTGA TCCAAGCCAA GTGAGCCATG GCACTGGCTT CACGTCCTTT

                    XhoI          XbaI
                    ~~~~~~        ~~~~
        G   L   L   K   L   E   *   *   S   R
1261    GGCTTACTCA AACTCGAGTG ATAATCTAGA
```

# Fig. 8

## Simultaneous Binding of 2H7scFv-CD154
## Fusion Proteins to CD20 and CD40

2H7scFv-CD154
Construct S4

2H7scFv-CD154
Construct L2
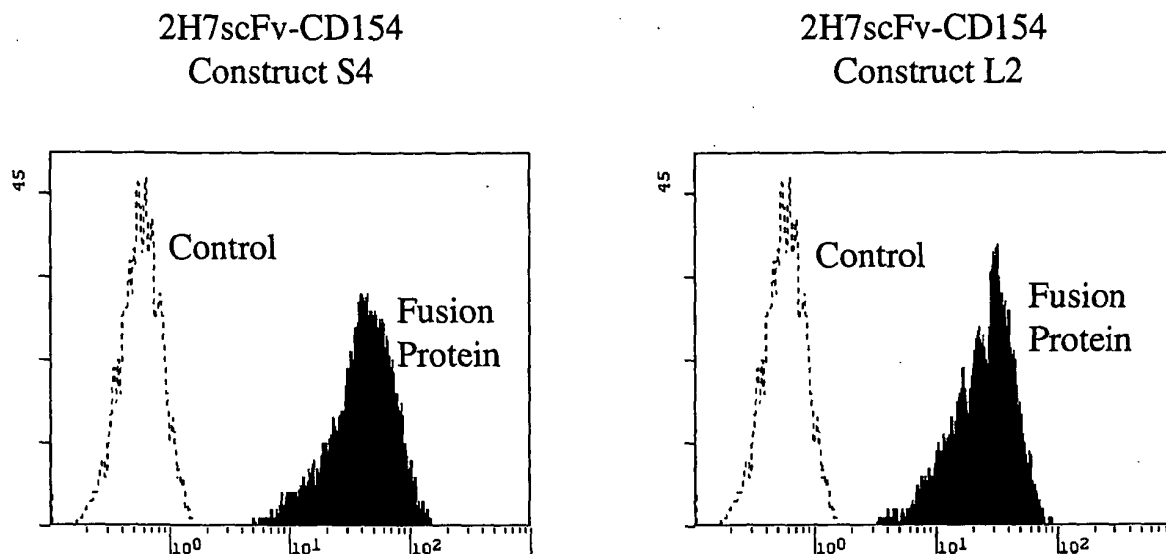


CD20 CHO cell targets + (control or fusion protein)
+ Biotin-CD40Ig + PE-SA

# Fig. 9

## Induction of Apoptosis Measured by Binding of Annexin V after incubation with 2H7scFv-CD154
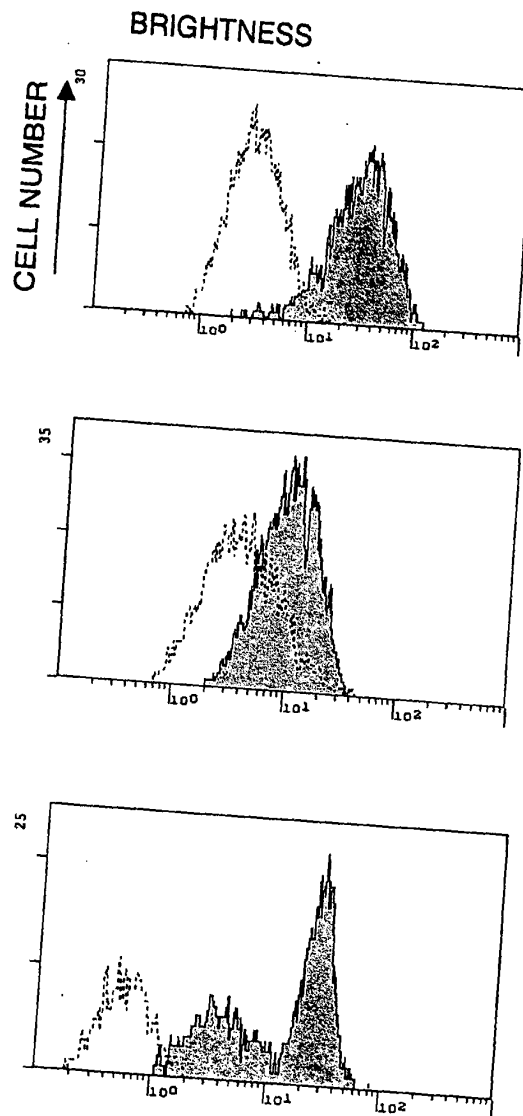


......control supernatant    ____2H7scFv-CD154 supernatant

Fig. 10



Proliferation of T51 B Cell Line After Incubation with 2H7
scFv-CD154 S4 or 2H7 scFv-CD154 L2 Constructs

# Fig. 11

## Schematic Representation of 2H7 scFvIg Constructs

2H7 scFvIgG (SSS-S)H WCH2 WCH3
OR 2H7 scFvIgG1 (SSS-S)H P238SCH2 WCH3 :     2H7 scFv

Human IgG CH2-CH3

| CH3 | CH2 | H | V$_H$ |
|-----|-----|---|-------|

| CH3 | CH2 | | V$_H$ |
|-----|-----|---|-------|

N434
H433

P331 / P331
K322
K320
E318 / E318
**N297/**N297
H310
I253
P238=WTG1C
P238→S=MCH2
G236 G237
L235
L234
E233

C229→S
C226→S
C220→S

[asp-gly$_3$-ser-{gly$_4$ser}$_2$]
peptide linker

2H7 scFv-IgAH G1-WCH2 WCH3:

2H7 scFv

Human IgG1 CH2-CH3

| CH3 | CH2 | huIgAhinge | V$_H$ |
|-----|-----|-----------|-------|

| CH3 | CH2 | huIgAhinge | V$_H$ |
|-----|-----|-----------|-------|

N434
H433

P331 / P331
K322
K320
E318 / E318
**N297/**N297
H310
I253
P238=WTG1C
P238→S=MCH2
G236 G237
L235
L234
E233

↑ =ADCC and FcR binding

| PASPSPTPPTSPSPTPPTSPVPQD |
|-------------------------|

↑ =Complement Fixation

# Fig. 12



**Non-reduced**                    **Reduced**

Figure 12: SDS-PAGE Analysis of CytoxB Derivatives. Purified fusion protein derivatives of CytoxB-scFvIg molecules and Rituximab were resuspended SDS sample buffer, boiled, loaded onto 10% Novex Tris-Bis gels (Invitrogen, San Diego, CA) and subjected to nonreducing (left panel) or reducing (right panel) SDS-PAGE electrophoresis at 175 volts. Two different molecular weight markers, BioRad prestained markers, and Novex Multimark molecular weight markers were also loaded onto each gel and the approximate size in kDa of each marker band is indicated along each side of the photographed gels. Gels were stained in Coomassie Blue stain and photographed with a SONY Mavica Digital camera. The mutant hinge forms of 2H7 scFvIgG1 migrate at approximately 70 kDa under both nonreducing and reducing conditions, indicating that these molecules are monomeric rather than dimeric in structure. The IgA hinge form of 2H7scFvIg migrates at approximately 75 kDa under reducing conditions, but migrates predominately as a dimer of 140 kDa with a fraction of the protein migrating at 75 kDa under nonreducing conditions. Under nonreducing conditions, rituximab migrates as a diffuse band of between 150 and 200 kDa. The heavy and light chains resolve into separate bands of approximately 32 and 50 kDa when rituximab is reduced and subjected to SDS-PAGE.

# Fig. 13
## ADCC Activity of CytoxB (2H7 scFvIg) Constructs.
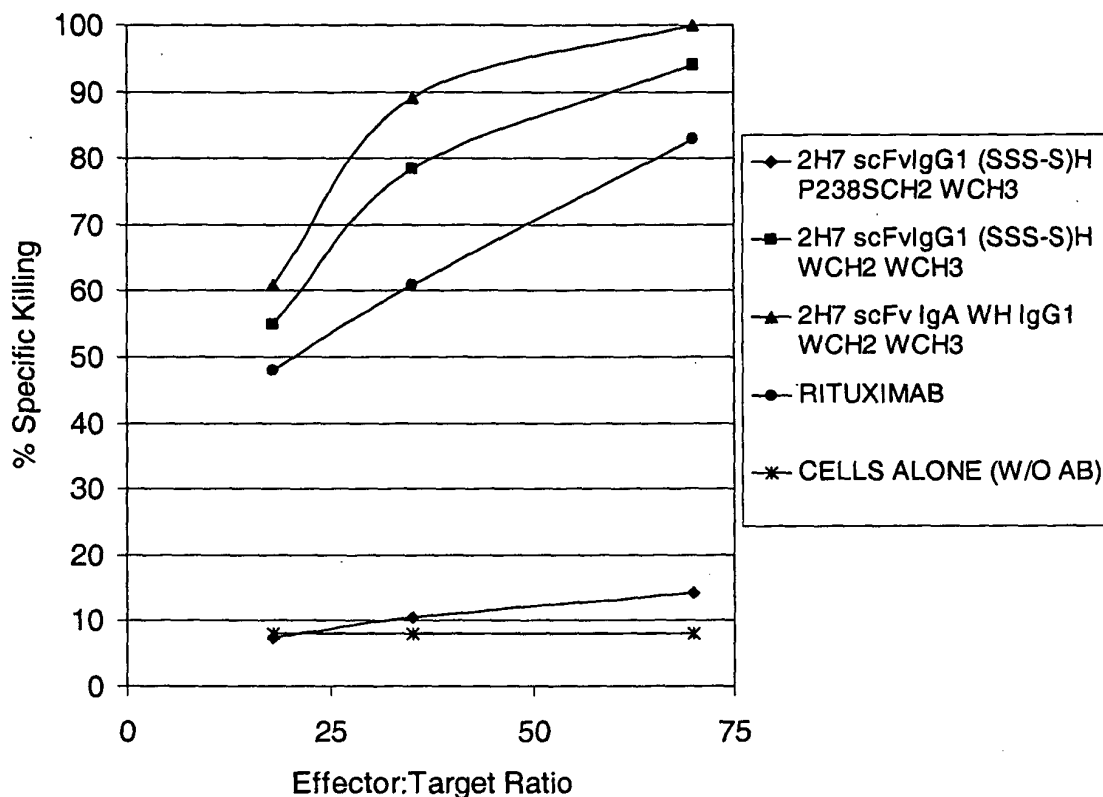


Figure 13: ADCC Activity of CytoxB Derivatives Compared to Rituximab. ADCC activity of CytoxB Derivatives or Rituximab was measured *in vitro* against BJAB B lymphoma cell line as target and using fresh human PBMC as effector cells. Effector to target ratios were varied as follows: 70:1, 35:1, and 18:1, with the number of BJAB cells per well remaining constant but varying the number of PBMC. Bjab cells were labeled for 2 hours with $^{51}$Cr and aliquoted at a cell density of $5 \times 10^4$ cells/well to each well of flat-bottom 96 well plates. Purified fusion proteins or rituximab were added at a concentration of 10 mg/ml, and PBMC were added at $9 \times 10^5$ cells /well (18:1), $1.8 \times 10^6$ cells/well (35:1), or $3.6 \times 10^6$ cells/well (70:1), in a final volume of 200 μl. Spontaneous release was measured without addition of PBMC or fusion protein, and maximal release was measured by the addition of detergent (1% NP-40) to the appropriate wells. Reactions were incubated for 4 hours, and 100 ml culture supernatant harvested to a Lumaplate (Packard Instruments) and allowed to dry overnight prior to counting cpm released on a Packard Top Count NXT Microplate Scintillation Counter.

# Fig. 14

## CDC of Cytox B (2H7 scFvIg) Constructs
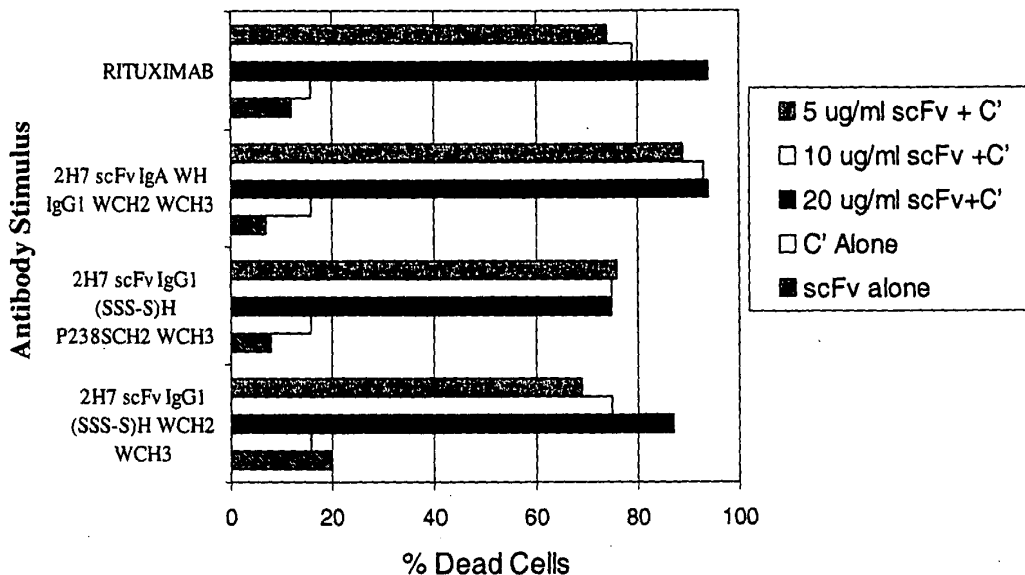


Figure 14: Complement Dependent Cytoxicity (CDC) Activity of CytoxB Derivatives
Compared to Rituximab. 2H7 scFvIgG1 (SSS-S)H WCH2 WCH3, 2H7 scFvIgG1 (SSS-
S)H WCH2 WCH3, and 2H7scFv IgA WH IgG1 WCH2 WCH3 derivatives and Rituximab
were compared for their ability to mediate complement dependent cytoxicity. Rabbit
complement (Pel-Freez) was diluted 1:10 and added to BJAB cells along with dilutions of
each antibody derivative (20 μg/ml, 10 μg/ml, and 5 μg/ml). Controls were also included
without addition of complement (C') or scFv derivative. Reactions were allowed to
continue for 1 hour, and cells from each well were then stained with trypan blue and the
cell viability counted using a hemacytometer. Data is graphed as % of dead cells/total
cells counted for each condition assayed.

# Fig. 15
## 2H7 (anti-CD20) scFv IgG1 (SSS-S)H WCH2 WCH3
## In Vivo Half Life

Anti-CD20 2H7 scFv IgG1 (SSS-S)H WCH2 WCH3
Standard Curve



microgram s/ml
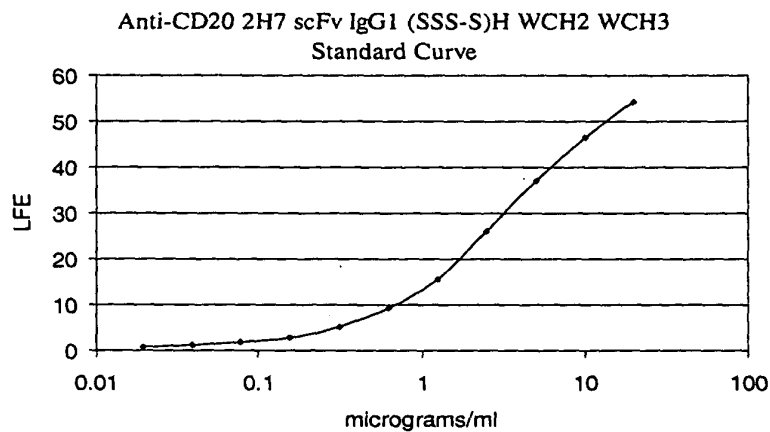
### Macaque A99314

| Day | Binding intensity (LFE) @1:50 dilution of serum | estimated concentration (µg/ml) |
|-----|---|---|
| -7 | 0.213 | <0.1 |
| 0 | 0.227 | <0.1 |
| 1 | 7.79 | 25.1 |
| 3 | 5.51 | 15.6 |
| 7 | 3.37 | 9.4 |
| 8 | 11.33 | 41.7 |
| 10 | 5.45 | 15.4 |
| 14 | 0.27 | <0.1 |

Injection #1 → 0
Injection #2 → 7

### Macaque F98081

| Day | Binding intensity (LFE) @ 1:50 dilution of serum | estimated concentration (µg/ml) |
|-----|---|---|
| -7 | 0.208 | <0.1 |
| 0 | 0.219 | <0.1 |
| 1 | 6.73 | 21.9 |
| 3 | 6.14 | 19.3 |
| 7 | 3.04 | 8.7 |
| 8 | 9.83 | 33.8 |
| 10 | 4.77 | 14.4 |
| 14 | 0.231 | <0.1 |

Injection #1 → 1
Injection #2 → 7

# Fig. 16

## B Cell Depletion in macaques mediated by Cytox B20 (2H7 scFv IgG1 (SSS-S)H WCH2 WCH3) Construct
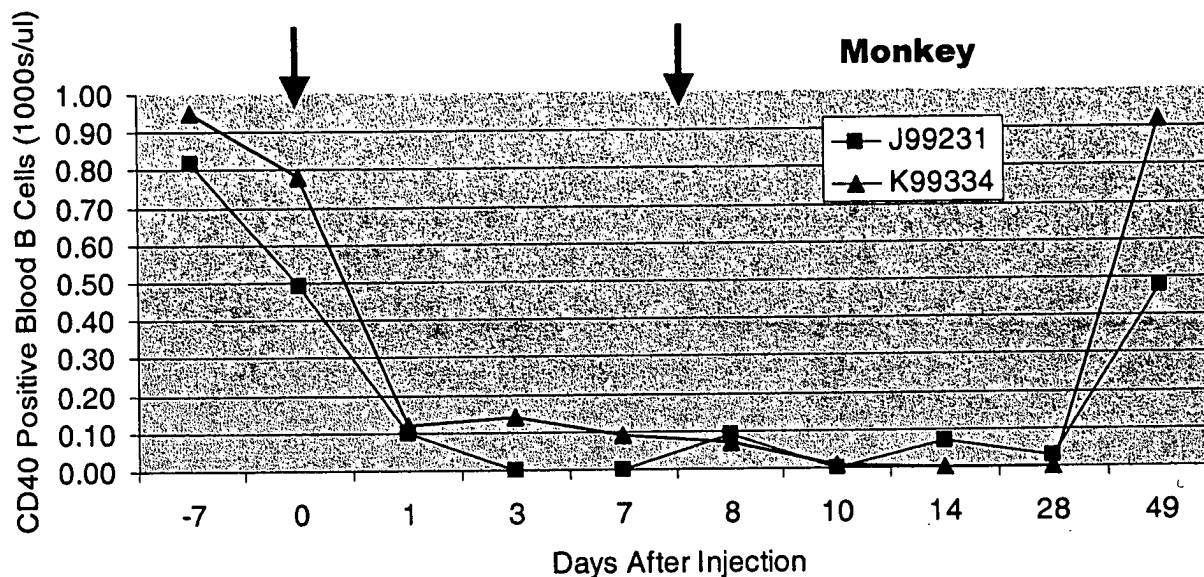


- **CytoxB20 injections of 6mg/kg yields 3 week B-cell depletion**
- **3-4 day half-life *in vivo***
- **CD20 saturation in lymph node B-cells at d14**
- **No first dose effects**
- **No anti-chimeric antibody development**

# Fig. 17
## Production Levels of HD37 scFvIg Constructs by CHO Cell Lines

### Standard Curve of HD37 scFvIg Construct Binding to B Cells



Mean LFE vs. Concentration (ug/ml)

Legend: —♦— HD37scFv IgA WH IgG1 WCH2 WCH3   —■— HD37scFv IgG1 (SSS-S)H WCH2 WCH3
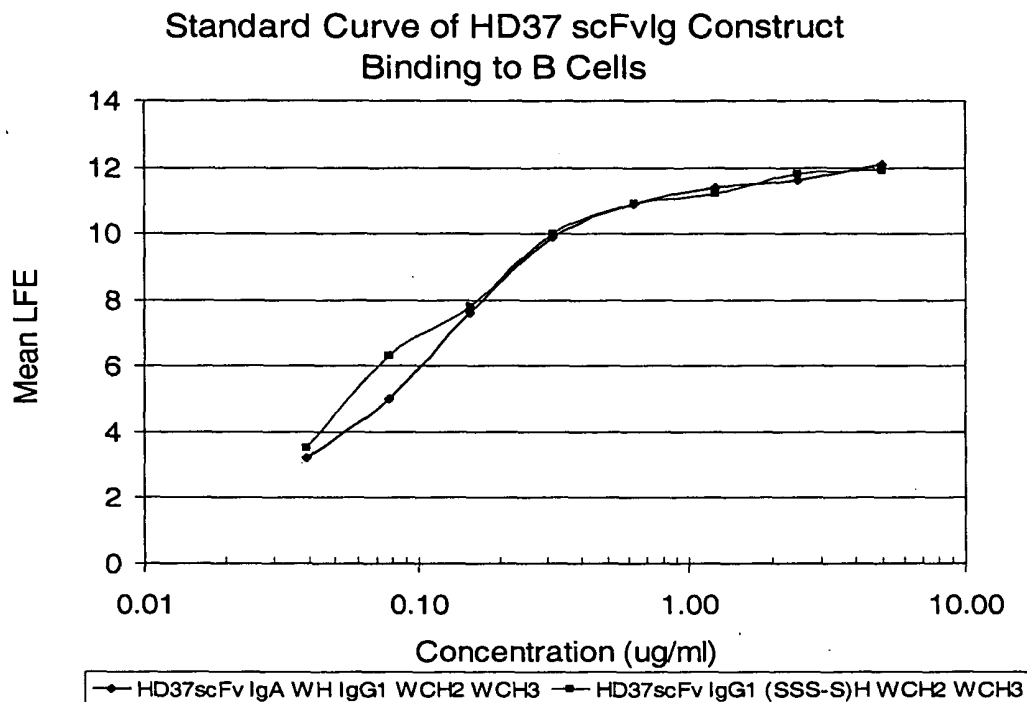
| Clone/Isolate | Mean LFE at 1:100 | Estimated Concentration |
|---|---|---|
| Bulk HD37 scFv | | |
| IgA WH IgG1 WCH2 WCH3 | 11.2 | > 60 ug/ml |
| 1B2 | 10.4 | >50 ug/ml |
| 6C5 | 10.5 | >50 ug/ml |
| 4B1 | 8.6 | >40 ug/ml |
| | | |
| Bulk HD37 scFv | | |
| IgG1 (SSS-S)H WCH2 WCH3 | 10.9 | > 50 ug/ml |
| 2G8 | 10.6 | > 50 ug/ml |
| 3F3 | 8.3 | >40 ug/ml |
| 3D9 | 11.1 | > 60 ug/ml |

# Fig. 18

## Production of L6 scFvIg constructs by CHO Cells



L6 scFvIg Standard Curves
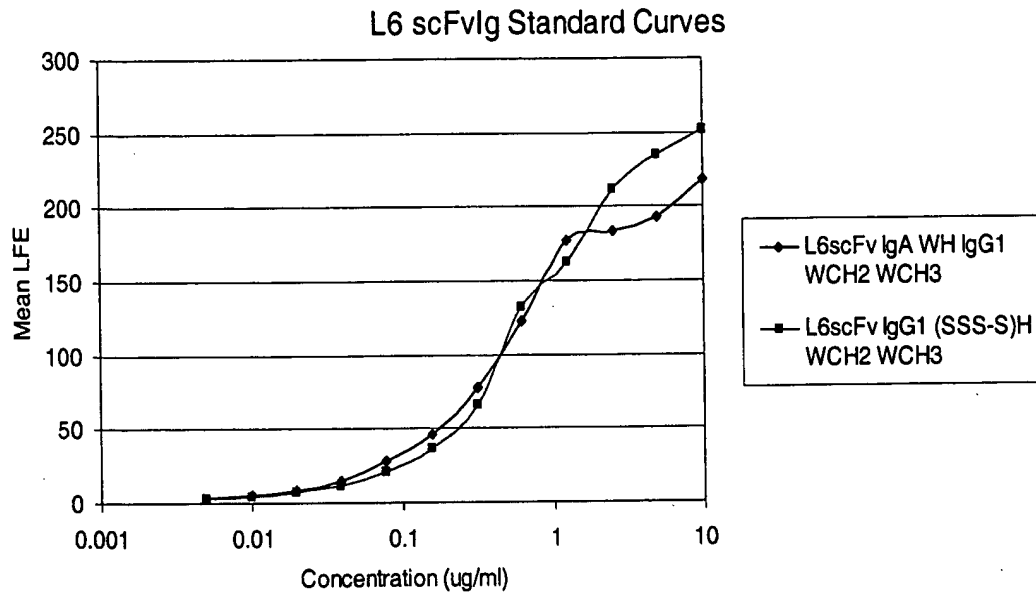
| Construct | Mean LFE 1:20 | Estimated Concentration |
|---|---|---|
| L6scFv IgA WH IgG1 WCH2 WCH3 unamplified CHO sup | 51.1 | 6.25 ug/ml |
| L6scFv IgG1(SSS-S)H WCH2 WCH3 unamplified CHO sup | 23.0 | 3.2 ug/ml |

# Fig. 19
# ADCC Activity of 2H7 scFvIg, G28-1 scFvIg, and HD37 scFvIg Constructs

## ADCC Activity of scFvs Targeted to B Cell Antigens

A. 2H7 (anti-CD20) scFv constructs
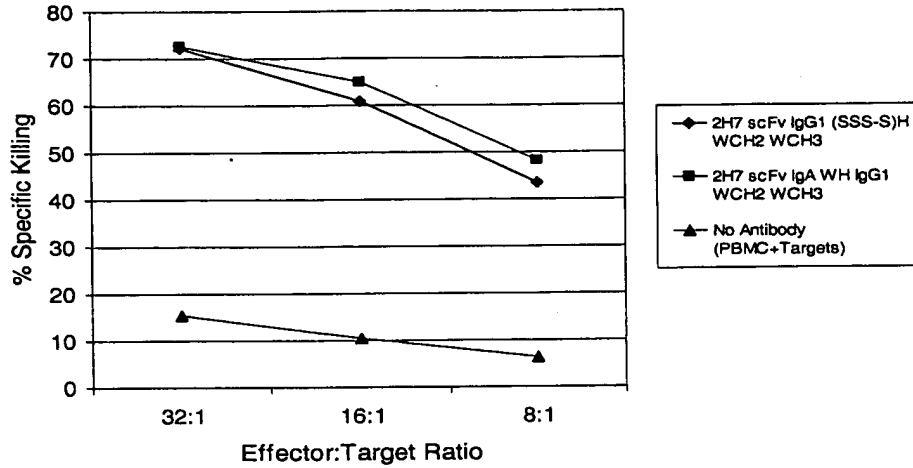


B. G28-1 (anti-CD37) scFv constructs
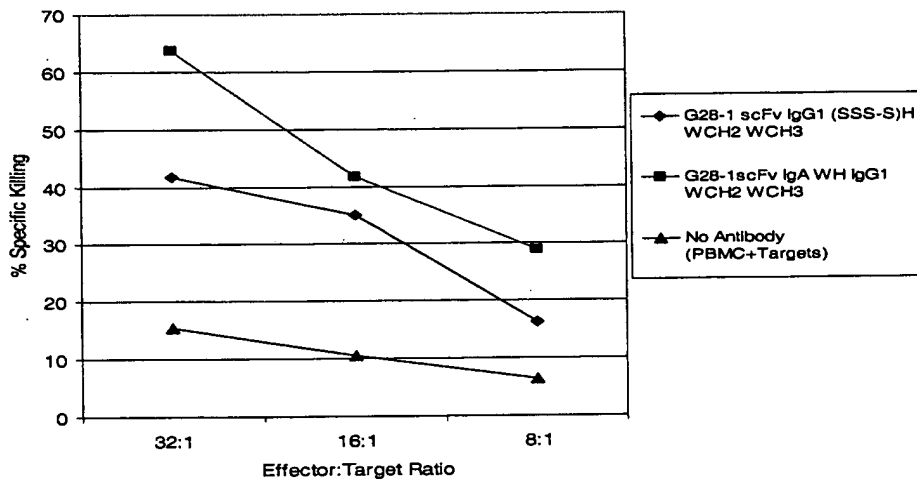


C. HD37 (anti-CD19) scFv constructs

# Fig. 20
# ADCC Activity of L6 scFvIg Constructs
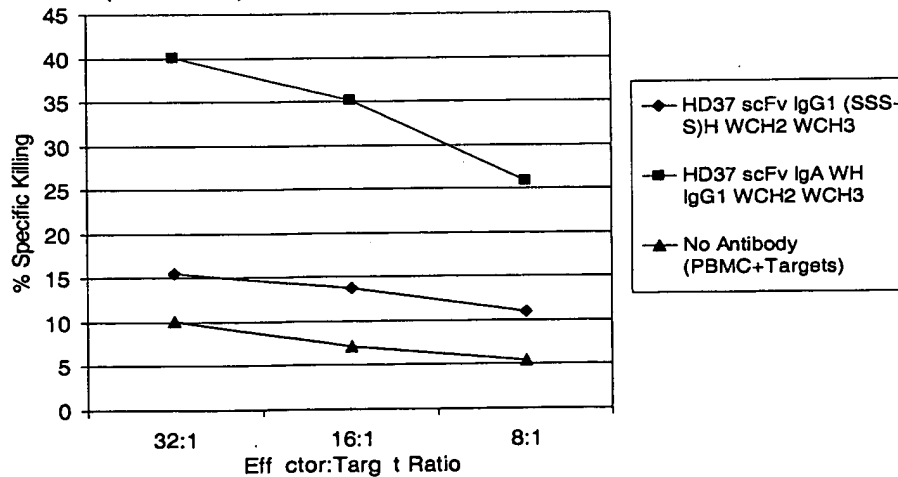
ADCC Activity of L6scFvIg Constructs with 2981 Targets
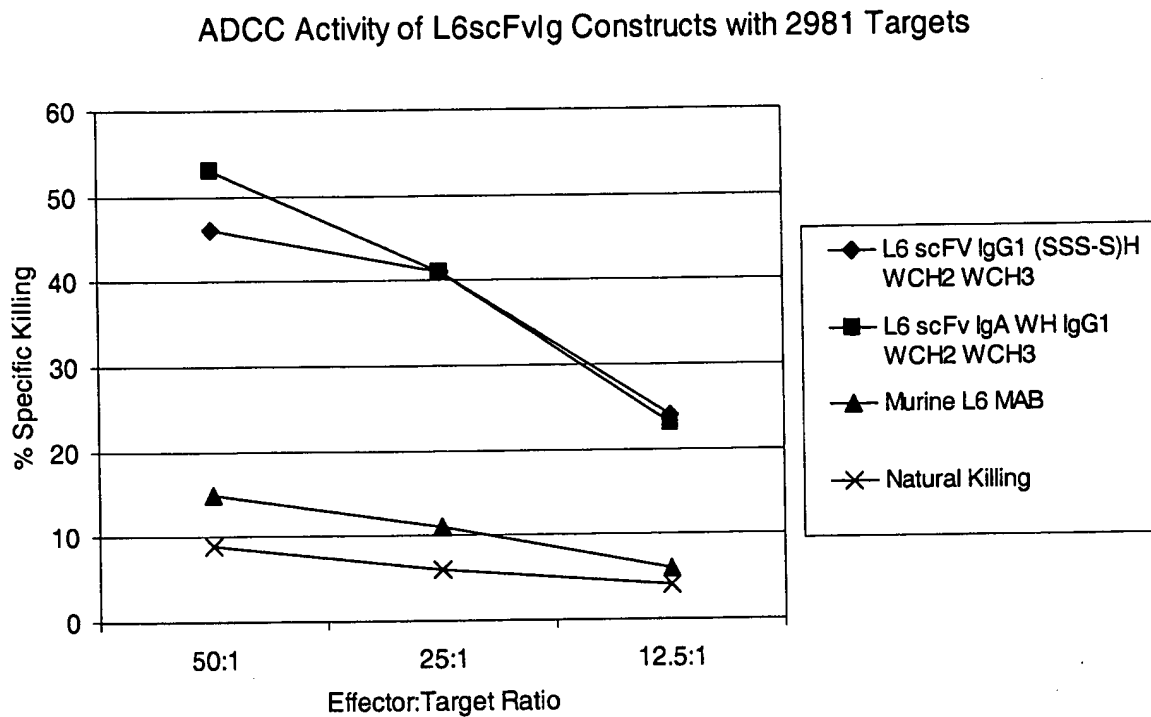
Fig. 21
SDS-PAGE Analysis of L6 and 2H7
scFvIg Fusion Proteins.
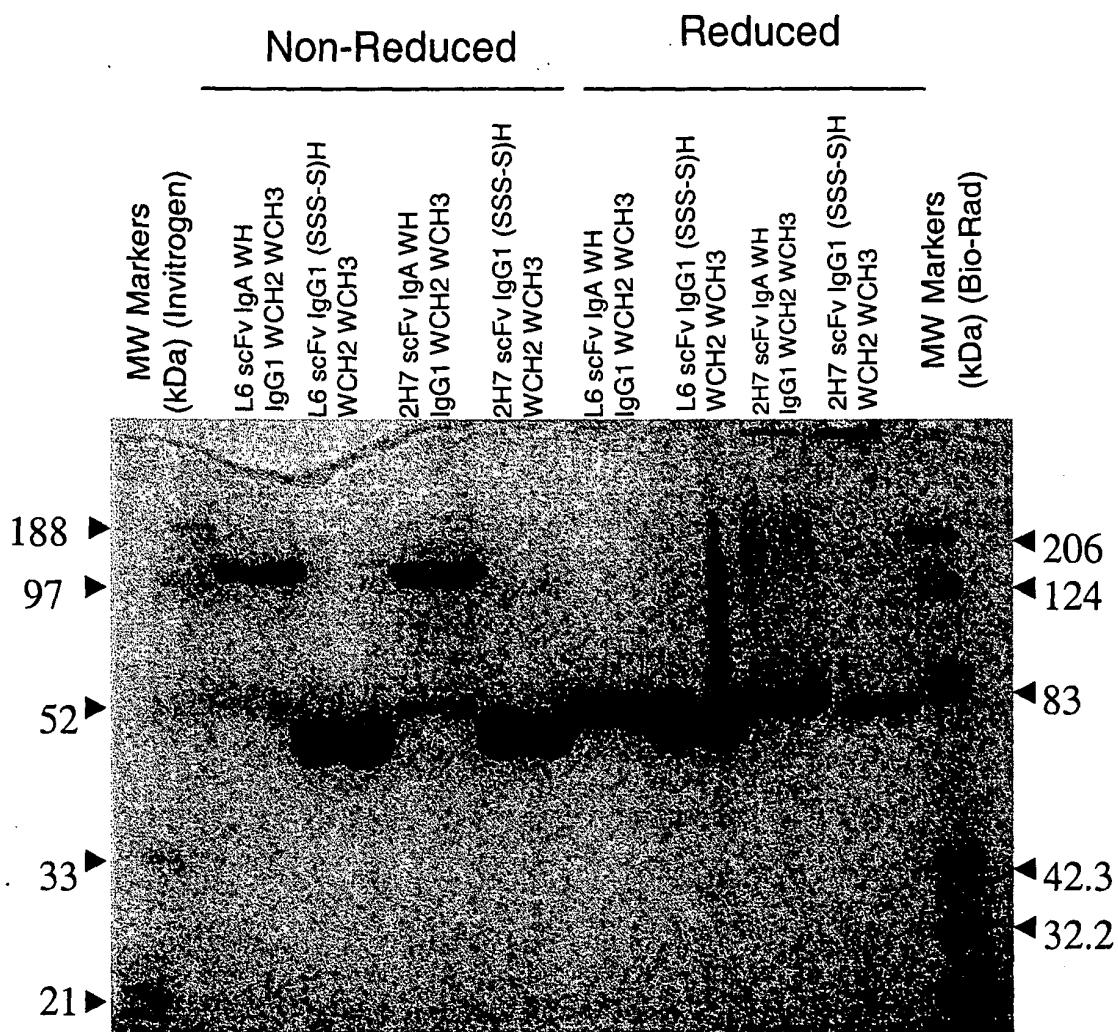
Fig. 22
SDS-PAGE Analysis of G28-1 and HD37
scFvIg Constructs.
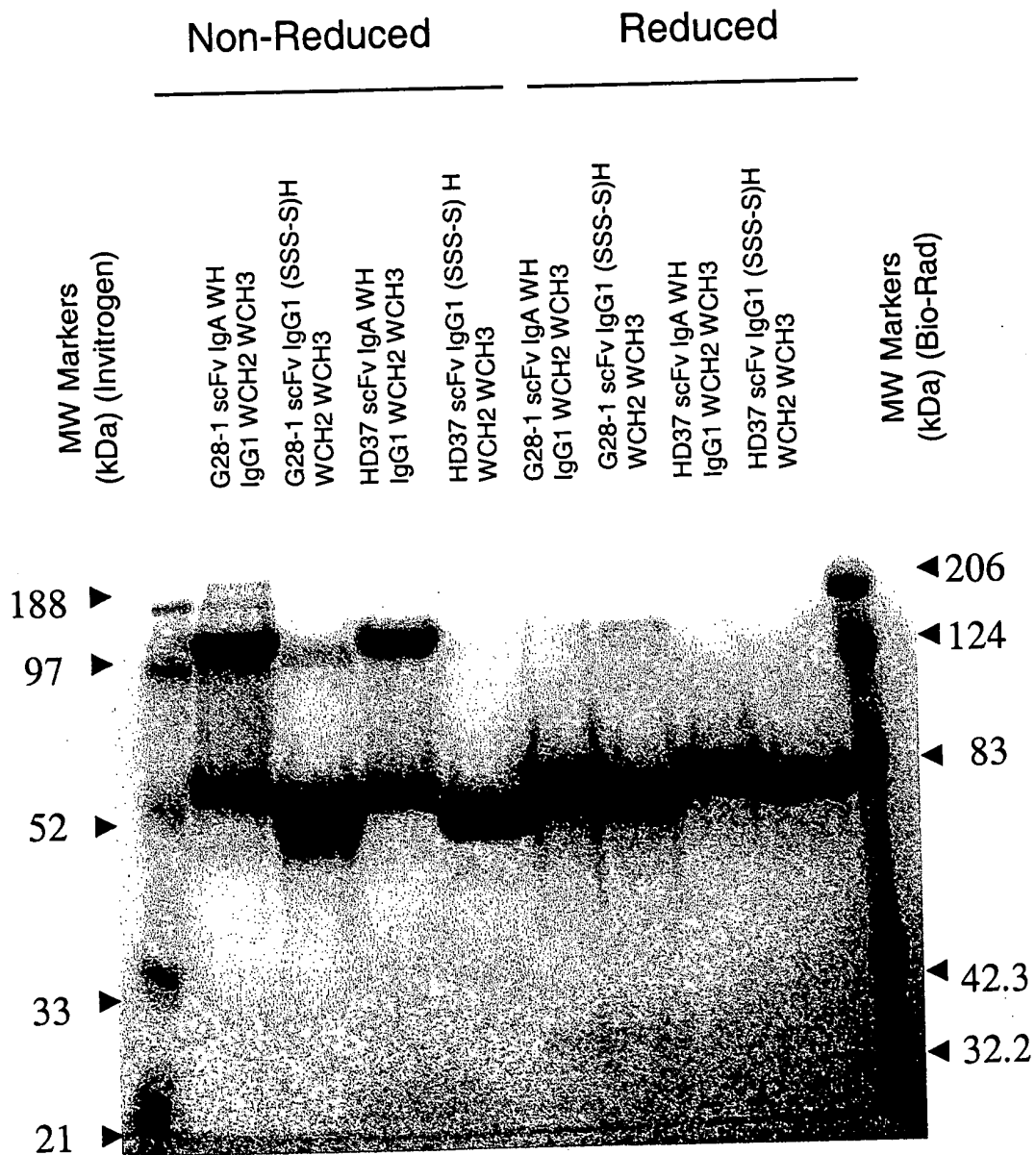
# Fig. 23
## Sequence alignment of human and llama Fc regions.

### HINGE          CH2→

```
Human IgG1: DQEPKSCDKT-----------HTCPPC PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
Llama IgG2: DQEPKTPKPQPQPQPQPNPTTESKCPKC PAPELLGGPSVFIFPPKPKDVLSISGRPEVTCVVVDVGQEDPEVSFNWYIDG
Llama IgG1: --EPHGG--------------CTCPQC PAPELPGGPSVFVFPPKPKDVLSISGRPEVTCVVVDVGKEDPEVNFNWYIDG
Llama IgG3: --AHHSEDPT-----------SKCPKC PGPELLGGPTVFIFPPKAKDVLSITRKPEVTCLWWTWVKKTLRSSSSWSVDD
```

```
VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLT
TAEVRANTRPKEEQFNSTYRVVSVLPIQHQDWLTGKEFKCKVNNKALPAPIEKTISKAKGQTREPQVYTLAPHREELAKDTVSVT
VEVRTANTKPKEEQFNSTYRVVSVLPIQHQDWLTGKEFKCKVNNKALPAPIERTISKAKGQTREPQVYTLAPHREELAKDTVSVT
TEVHTAETKPKEEQFNSTYRVVSVLPIQHQDWLTGKEFKCKVNNKALPAPIERTISKAKGQTREPQVYTLAPHREELAKDTVSVT
```

```
CLVKGFYPSDIAVEWESNGQPEN--NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
CLVKGFYPPDINVEWQRNGQPESXGTYATTPPQLDNDGTYFLXSKXSVGKNTWQQGETFTCVVMHEALHNHYTQKSITQSSGK
CLVKGFYPADINVEWQRNGQPESEGTYANTPPQLDNDGTYFLYSRLSVGKNTWQRGETLTGVVMHEALHNHYTQKSITQSSGK
CLVKGFFPADINVEWQRNGQPESEGTYANTPPQLDNDGTYFLYSKLSVGKNTWQQGEVFTCVVMHEALHNHSTQKSITQSSGK
```

# Fig. 24

## Fig. 25



Llama Tails Binding Assay with CD20 CHO Cells
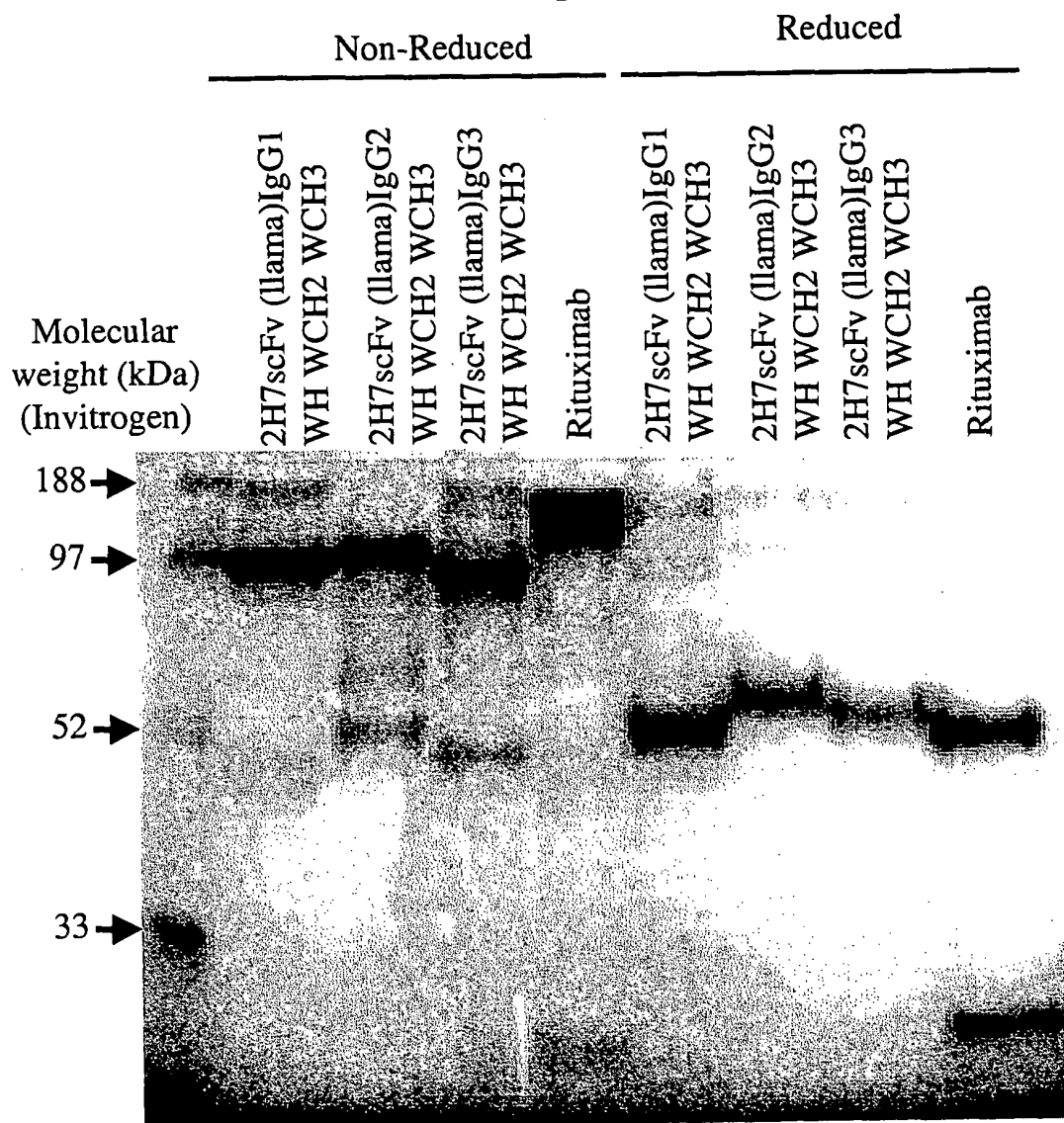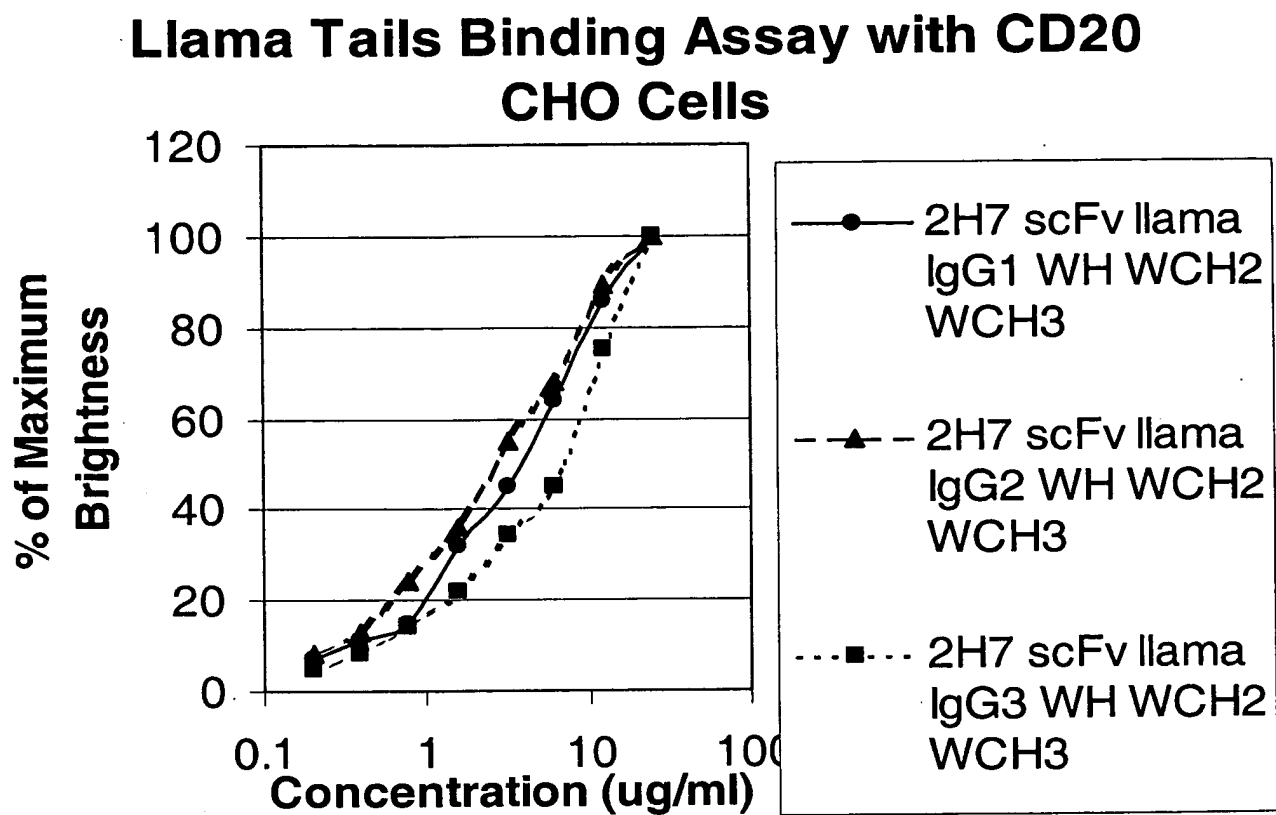
# Fig. 26

## 2H7 scFvIg Llama Tails binding Assay with CD20 CHO Cells



Legend:
- - - ◆ - - - 2H7 scFv llama IgG1 WH WCH2 WCH3
——■—— 2H7 scFv llama IgG2 WH WCH2 WCH3
——▲—— 2H7 scFv llama IgG3 WH WCH2 WCH3
——■—— 2H7 scFv human IgG1 (SSS-S)H WCH2 WCH3

Y-axis: % KILLING
X-axis: CONCENTRATION (ug/ml)

## Fig. 27
## ADCC Assay with BJAB Targets
## and Human PBMC Effectors
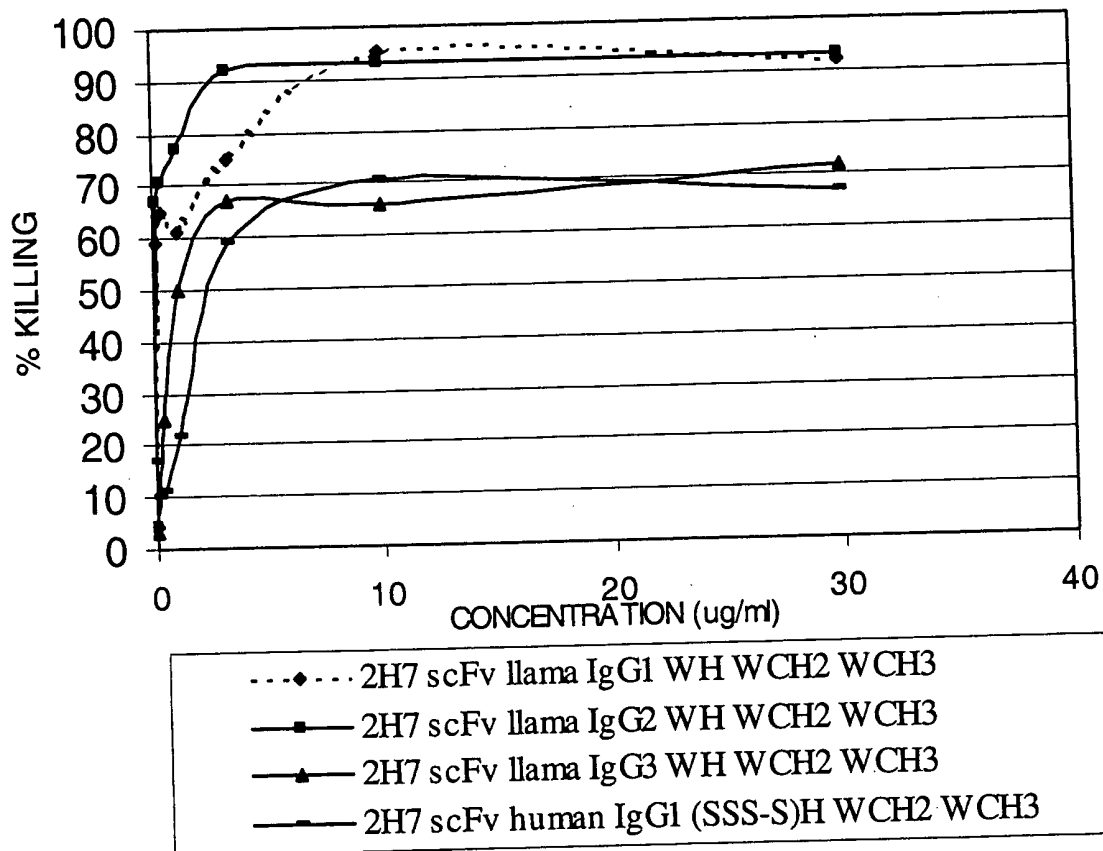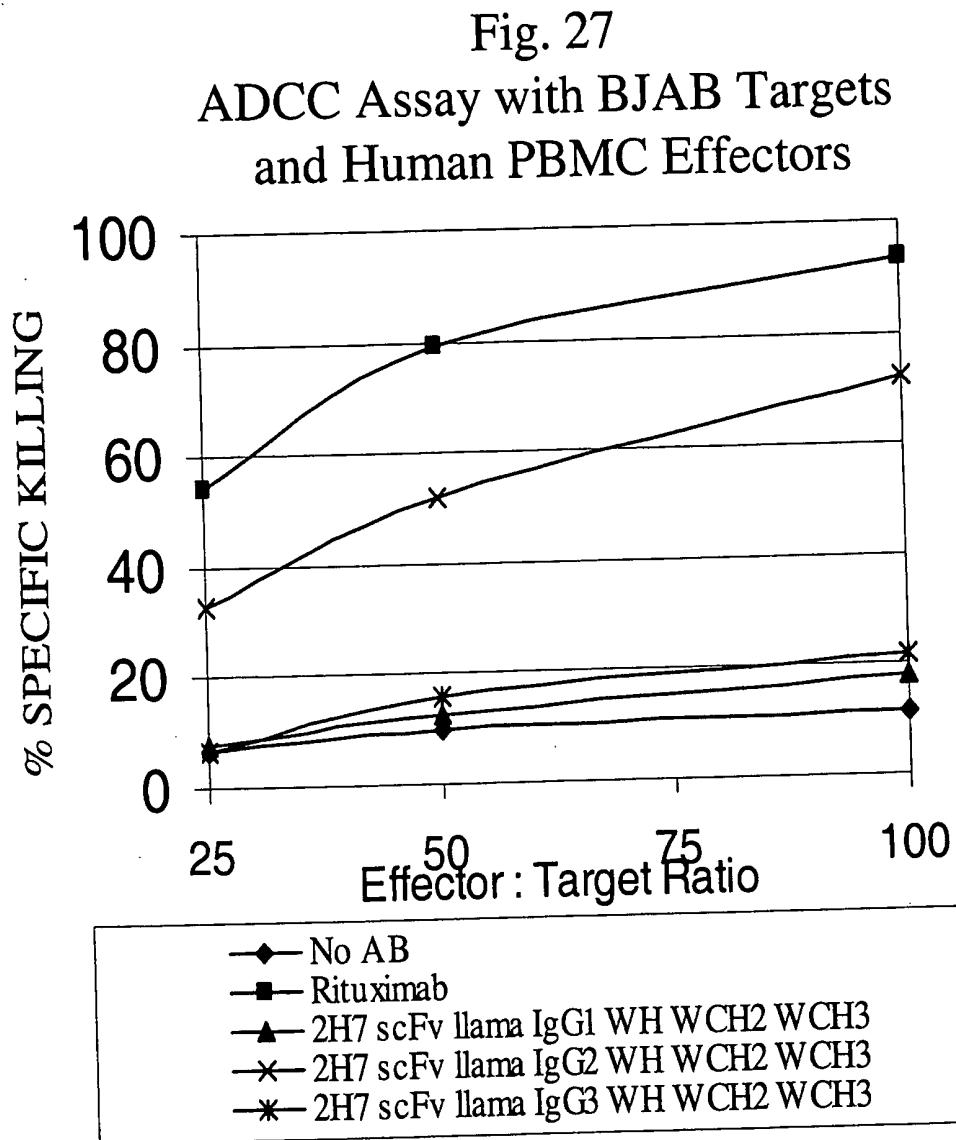
# Fig. 28

## ADCC Assay with BJAB Cells
## And Llama PBMC Effectors

# Fig. 29

Complement Mediated Cytotoxicity Against Reh
Cell Lines Expressing Surface ScFvIg Constructs



Surface scFvIg and Effector Functions
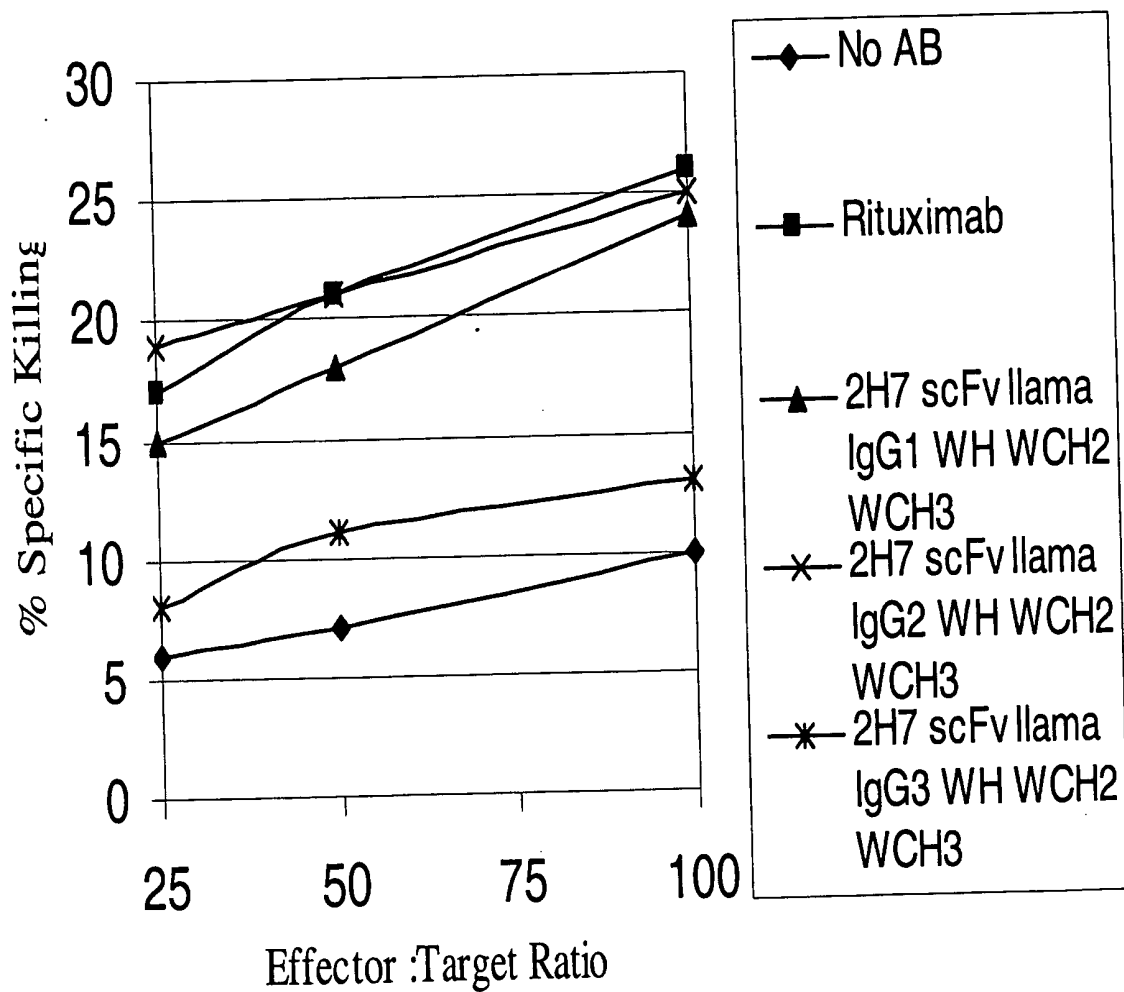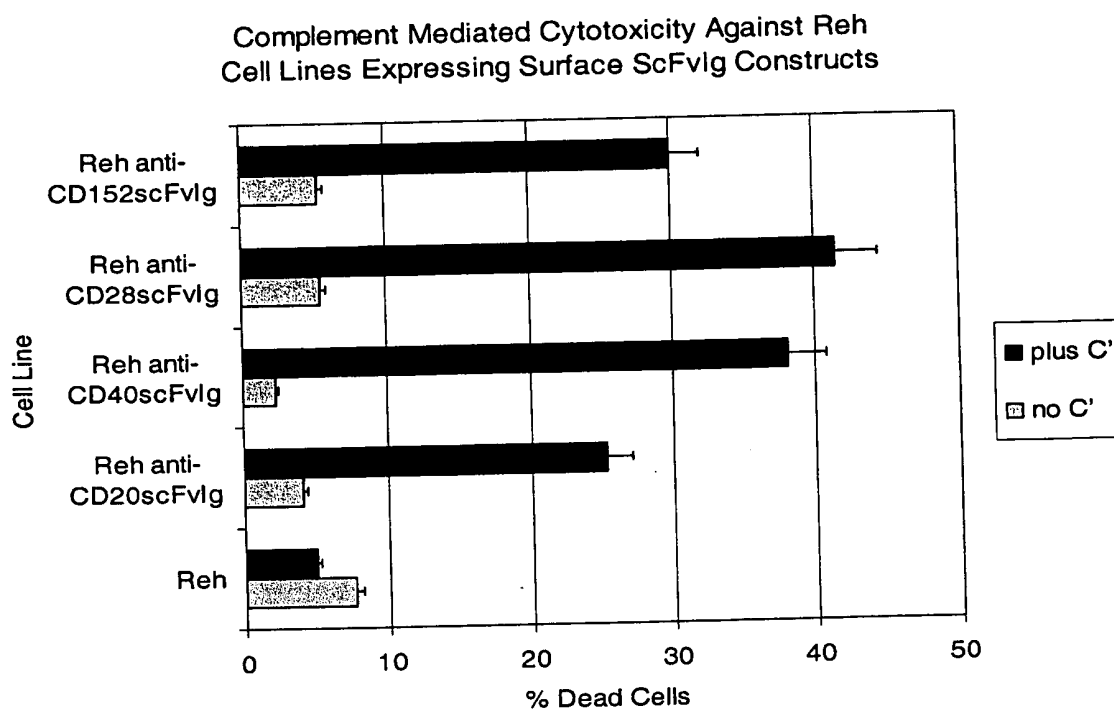
# Fig. 30

## ADCC Activity of Cell Surface Expressed ScFvIg Constructs



Legend:
- ◆ Reh
- ■ RehCD80.10
- ▲ Reh anti-hCD20
- ✳ Reh antihCD40
- ✱ Reh anti-hCD28
- ● Reh anti-hCD152
- ＋ Reh anti-mCD3
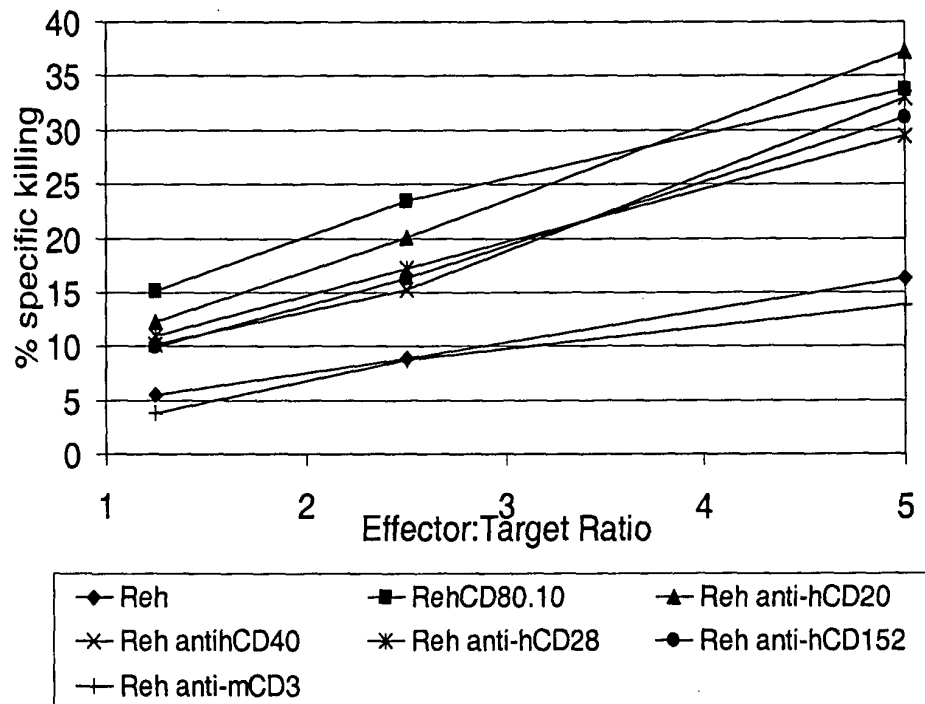
Surface scFvIg and Effector Functions

# Fig. 31
## Ig Constructs and Nomenclature:

| Name Identifier | Hinge Sequence | CH2 Sequence | CH3 Sequence |
|---|---|---|---|
| hIgG1 (CCC-P)H WCH2 WCH3 | IgG1 WT Hinge (CCC-P) | Wild Type CH2 | Wild Type CH3 |
| hIgG1 (SSS-S)H WCH2 WCH3 | IgG1 Mutant Hinge (SSS-S) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| VH L11S hIgG1 (SSS-S)H WCH2 WCH3 | IgG1 Mutant Hinge (SSS-S) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| IgG1 (SSC-S)H WCH2 WCH3 | IgG1 Mutant Hinge (SSC-S) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| IgG1 (SCS-S)H WCH2 WCH3 | IgG1 Mutant Hinge (SCS-S) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| IgG1 (CSS-S)H WCH2 WCH3 | IgG1 Mutant Hinge (CSS-S) | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| IgG1 (SSS-S)H P238S CH2 WCH3 | IgG1 Mutant Hinge (SSS-S) | Mutant CH2 (IgG1) Pro$\rightarrow$Ser 238 | Wild type CH3 (IgG1) |
| IgA WH hIgG1 WCH2 WCH3 | IgA Hinge | Wild type CH2 (IgG1) | Wild type CH3 (IgG1) |
| IgA WH IgA WCH2 WCH3 | IgA Hinge | Wild type CH2 (IgA) | Wild type CH3 (IgA) |
| IgA WH IgA WCH2 T4CH3 | IgA Hinge | Wild type CH2 (IgA) | Truncated CH3 (IgA) Missing 4 aa at COOH |

# Fig. 32

CDC Activity of CTLA4Ig Constructs against Reh and Reh CD80
Transfected Cells

# Fig. 33



A. Natural Killing

Reh
Reh CD80

Effector:Target Ratio

B. ADCC Mediated by CTLA4 hIgG1 (SSS-S)H P238SCH2 WCH3

Reh + CTLA4 hIgG1 (SSS-S)H P238SCH2 WCH3

Reh CD80 + CTLA4 hIgG1 (SSS-S)H P238SCH2 WCH3

Effector:Target Ratio

C. ADCC Mediated by CTLA4 hIgG1 (SSS-S)H WCH2 WCH3

Reh + CTLA4 hIgG1 (SSS-S)H WCH2 WCH3

Reh CD80 + CTLA4 hIgG1 (SSS-S)H WCH2 WCH3

Effector:Target Ratio

# Fig. 34

## Binding of 2H7 scFvIg Constructs
## with Alternative Tails to CD20 CHO Cells
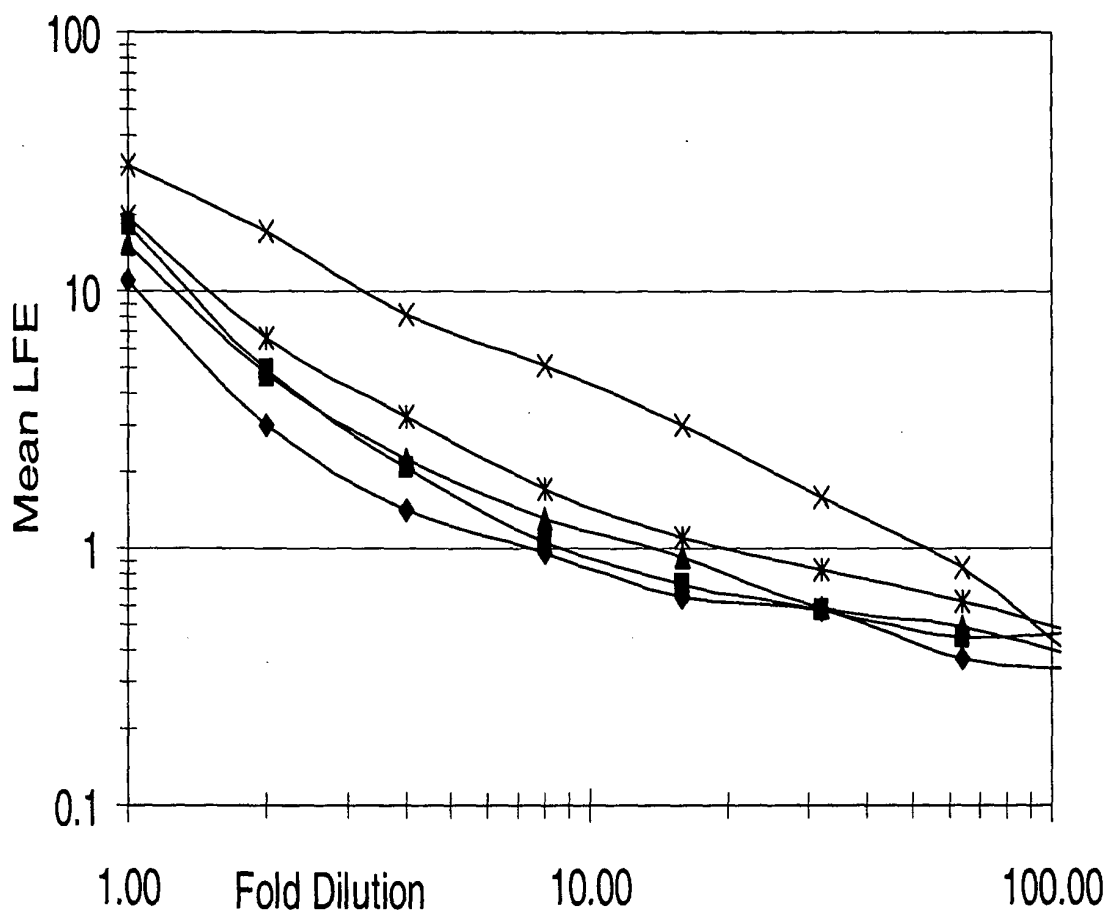


Legend:

◆ 2H7 scFv hIgG1 (CCC-P)H WCH2 WCH3    ■ 2H7 scFv hIgG1 (CSS-S)H WCH2 WCH3

▲ 2H7 scFv hIgG1 (SCS-S)H WCH2 WCH3    ✳ 2H7 scFv hIgG1 (SSC-S)H WCH2 WCH3

✳ 2H7 scFv VH L11S hIgG1(CCC-P)H WCH2 WCH3

# Fig. 35
## Immunoblot Analysis of protein immunoprecipitates from COS transfections of 2H7 scFvIg Constructs
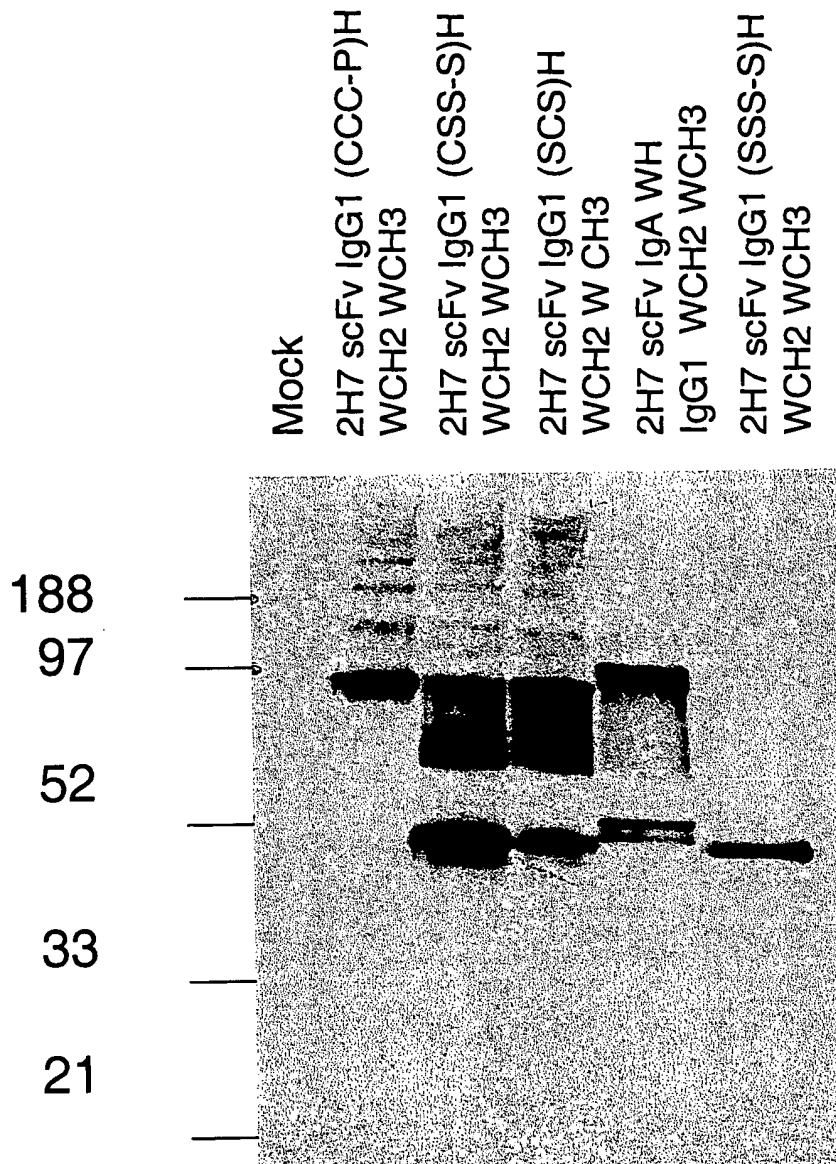
# Fig. 36

Binding to CD20 CHO cells by constructs
that link anti-CD20 scFv to IgA Fc Domains



goat anti-human IgA-FITC only

Co-transfected 2H7 scFv
IgA WH WCH2 WCH3
and  J-Chain + goat anti-
human IgA-FITC

2H7 scFv IgA WH
WCH2 T4CH3 + goat
anti-human IgA-FITC

Events

FL1 LOG

Brightness
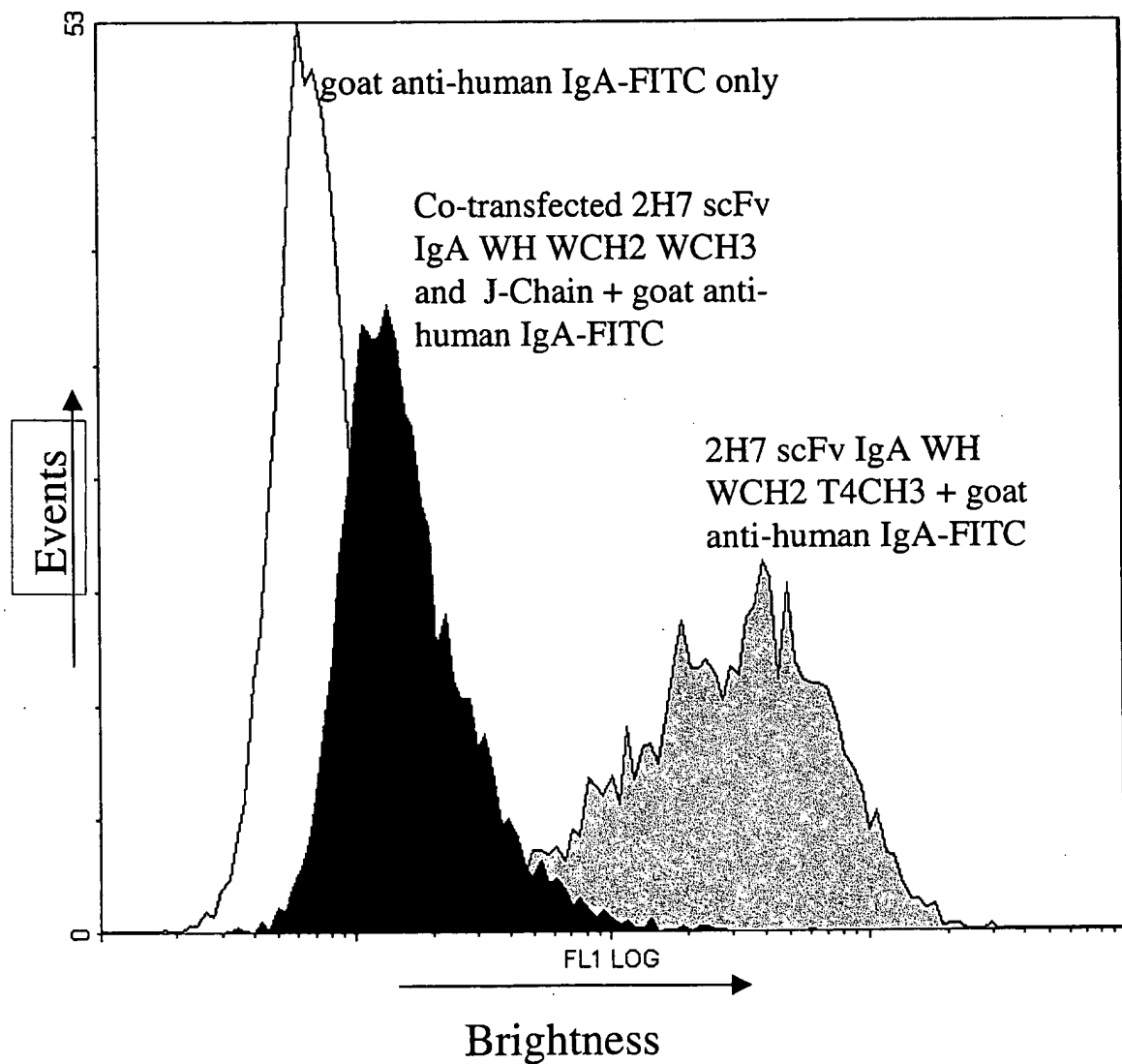
# Fig. 37

## Titration of CD20 specific scFvIg Constructs for ADCC Activity Using Whole Blood Effectors



Legend:
- ◆ 2H7 scFv IgG1 (SSS-S)H WCH2 WCH3
- ■ 2H7 scFv IgA WH IgG1 WCH2 WCH3
- ▲ 2H7 scFv IgG1 (CCC-P)H WCH2 WCH3
- ✳ 2H7 scFv IgG1 (SCS-S)H WCH2 WCH3

X-axis: Concentration (ug/ml)
Y-axis: % specific killing
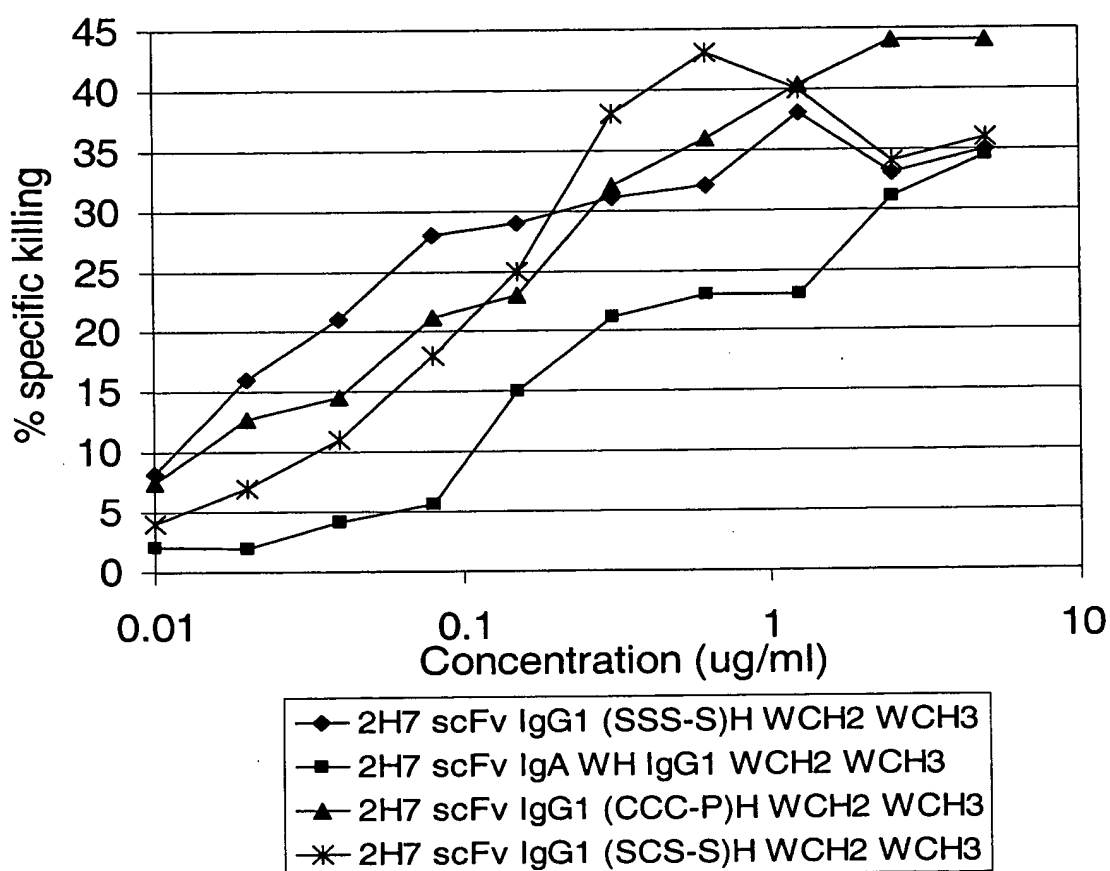
# Fig. 38

ADCC Assay of anti-CD20 constructs with alternative tails
(Whole Blood Effectors / BJAB Targets
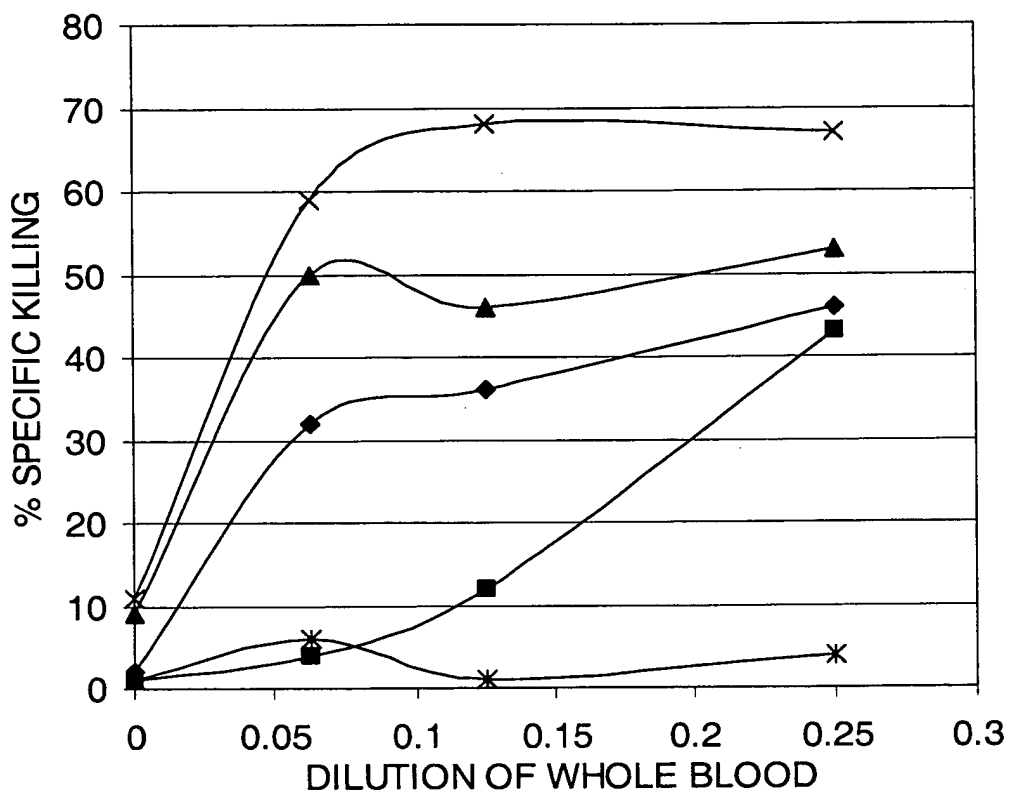


Legend:
- ◆ 2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3
- ■ 2H7 scFv IgA WH WCH2 T4CH3
- ▲ 2H7 scFv hIgG1 (SCS-S)H WCH2 WCH3
- ✕ 2H7 scFv hIgG1 (CCC-P)H WCH2 WCH3
- ✻ Effectors Only

# Fig. 39
## ADCC Assay of Anti-CD20 scFvIg Constructs
## Using Different Effector Populations Against BJAB Targets

A. (PBMC effectors/BJAB Targets)



-◆- 2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3
-■- 2H7 scFv IgA WH WCH2 T4CH3
-▲- Effectors Only

B. WHOLE BLOOD EFFECTORS/BJAB TARGETS



-◆- 2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3  -■- 2H7 scFv IgA WH WCH2 T4CH3
-▲- Effectors Only
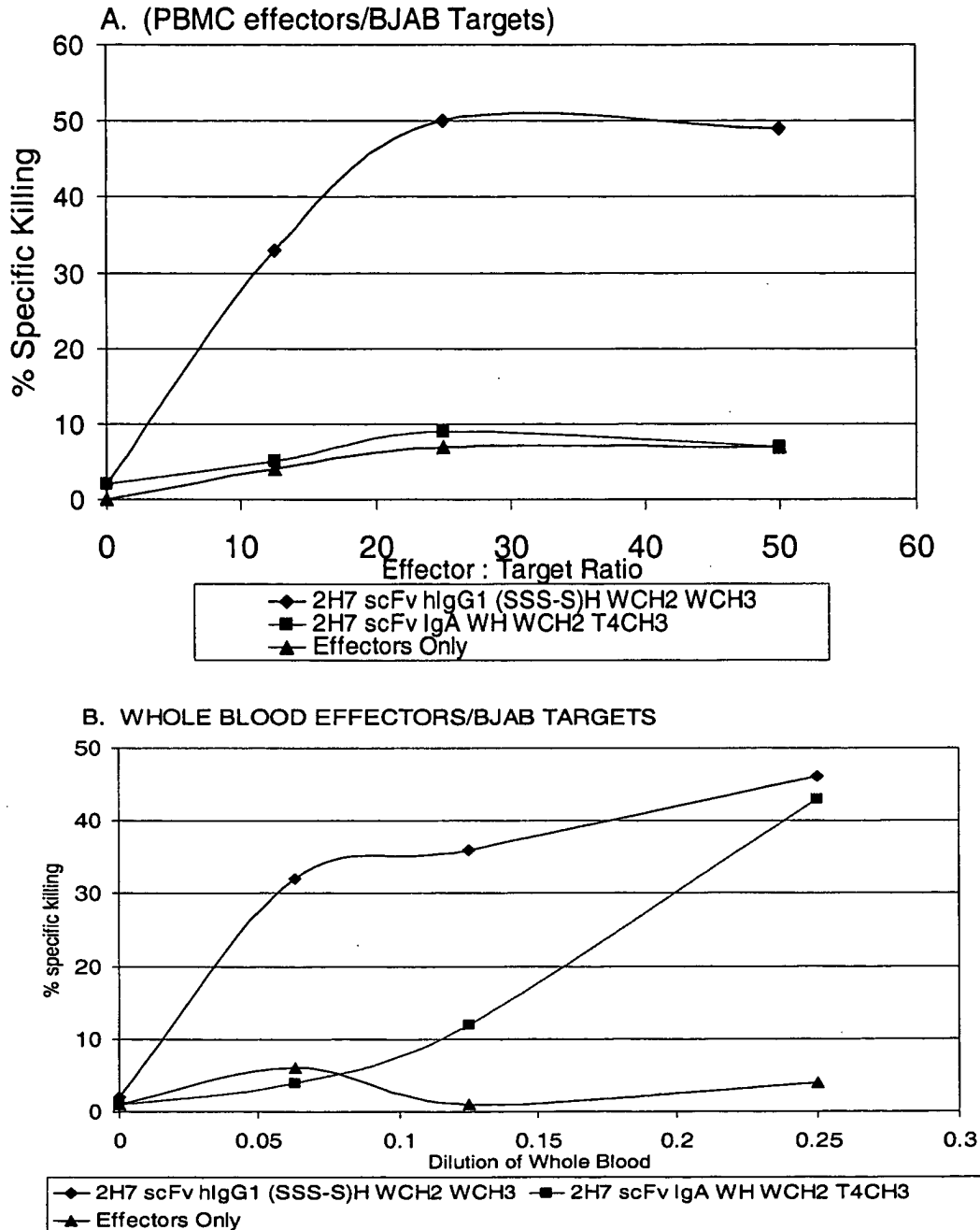
# Fig. 40

## Immunoblot of 2H7 scFv Ig constructs from COS
## Transfections (1 µl/well) compared to a Concentration Standard

# Figures 41A, 41B and 41C

**A.**



**B.**



**Sheet 45 of 53**

**C.**

# Fig. 42



Days Post Tumor Challenge
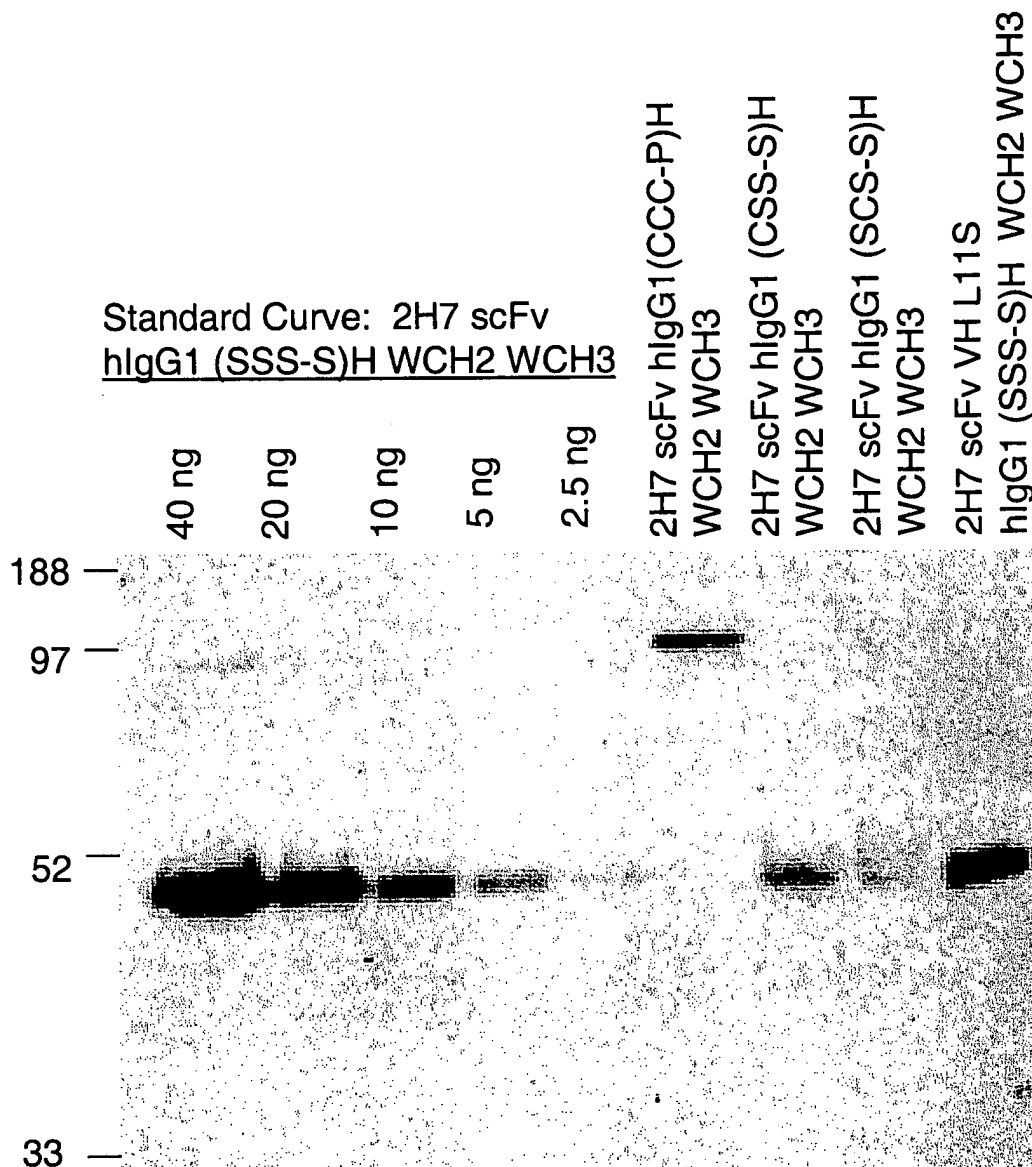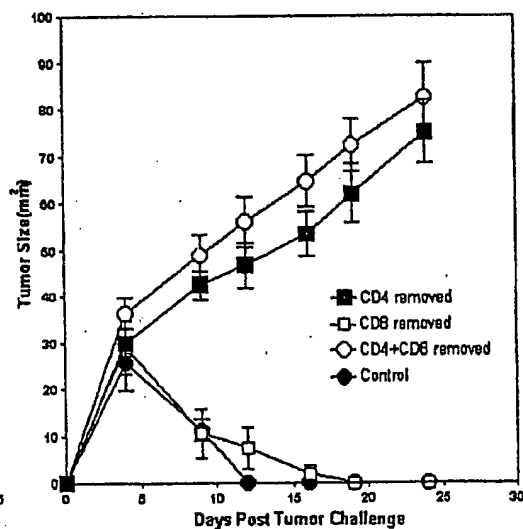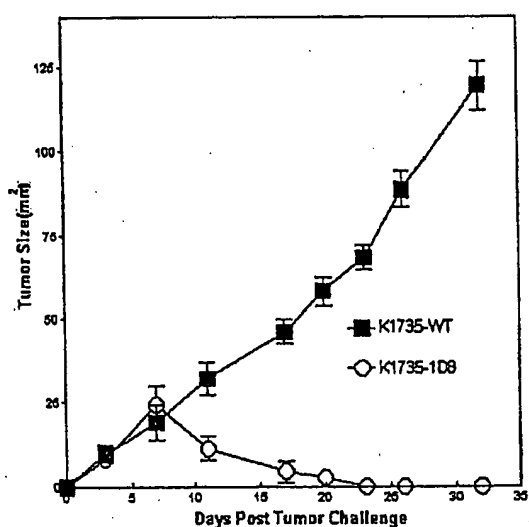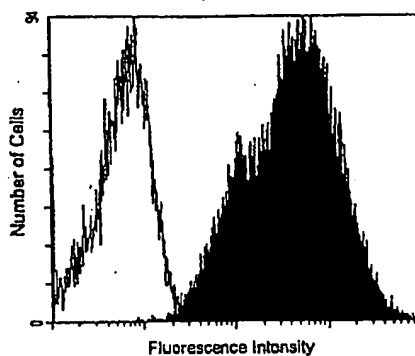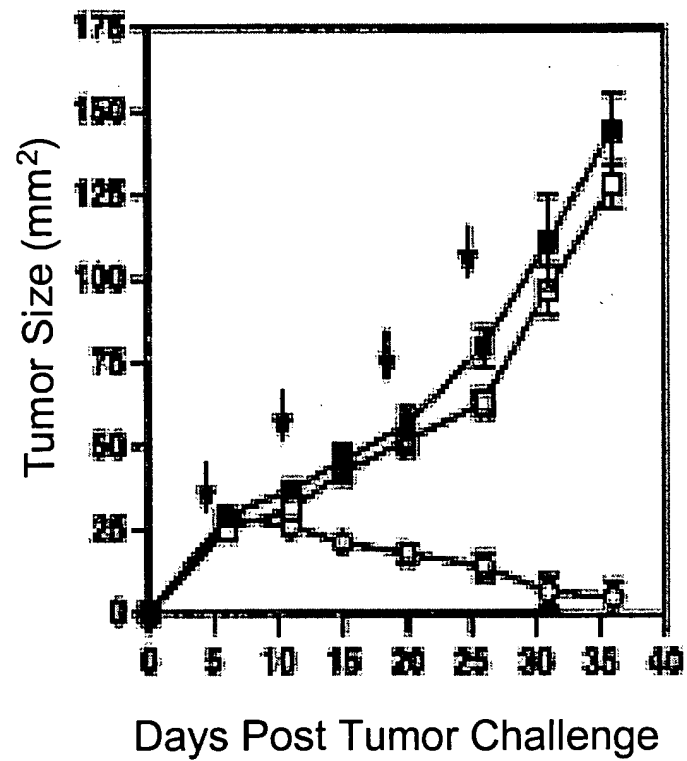
# Fig. 43

Mixtures of K1735-WT and K1735-1D8 transfected tumor lines
inhibit tumor outgrowth in C3H mice
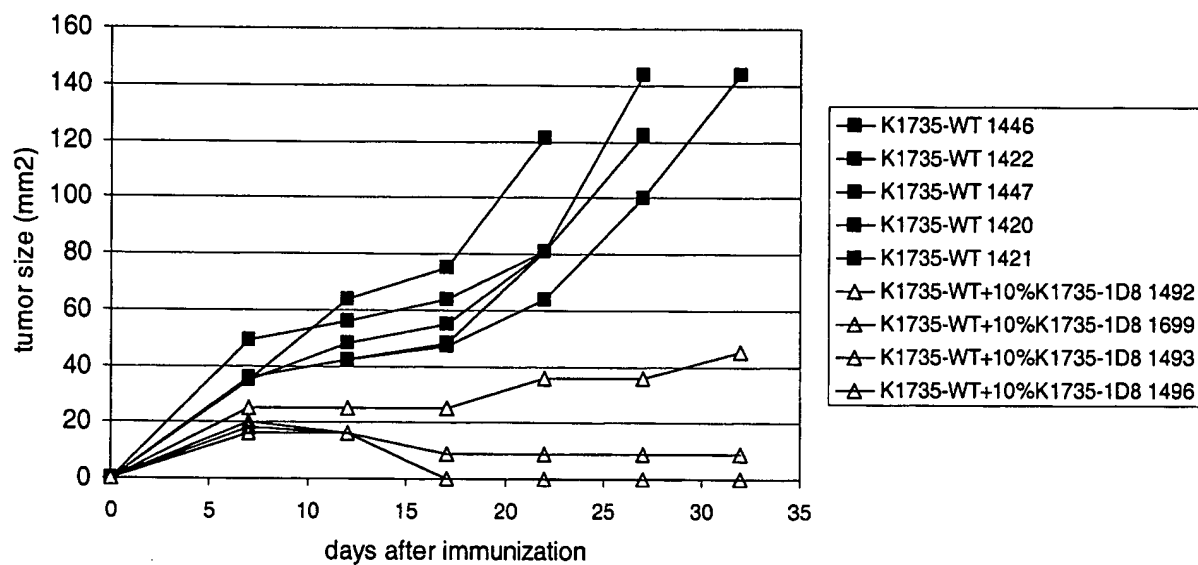
# Fig. 44

Expression of anti-mouse CD137 (1D8) scFv-hIgG1 (SSS-S)H P238SCH2 WCH3
On the surface of panned Ag104-1D8 Transfected Tumor Cells



Events

Brightness
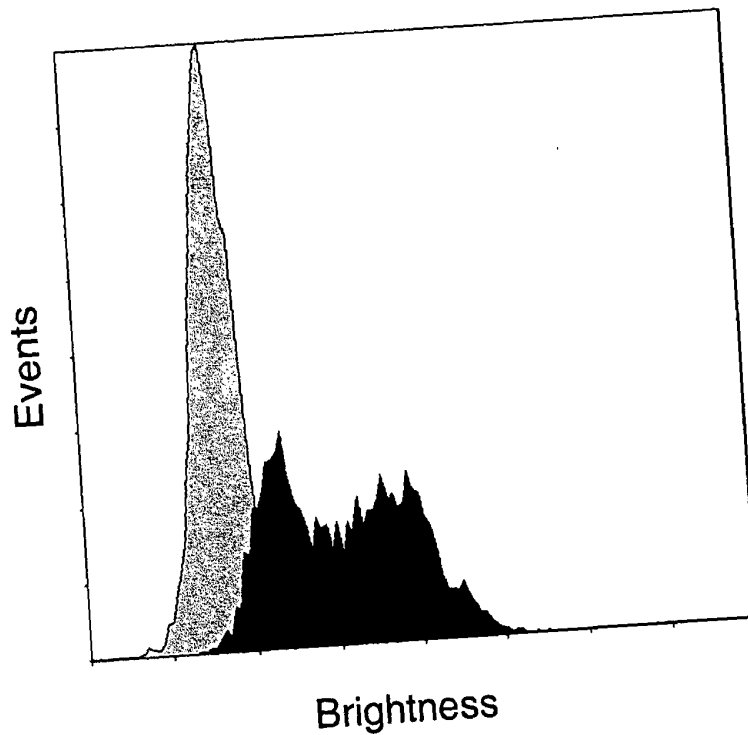
# Fig. 45

## Coomassie Stained SDS-PAGE Gel of 2H7 scFv Ig Constructs
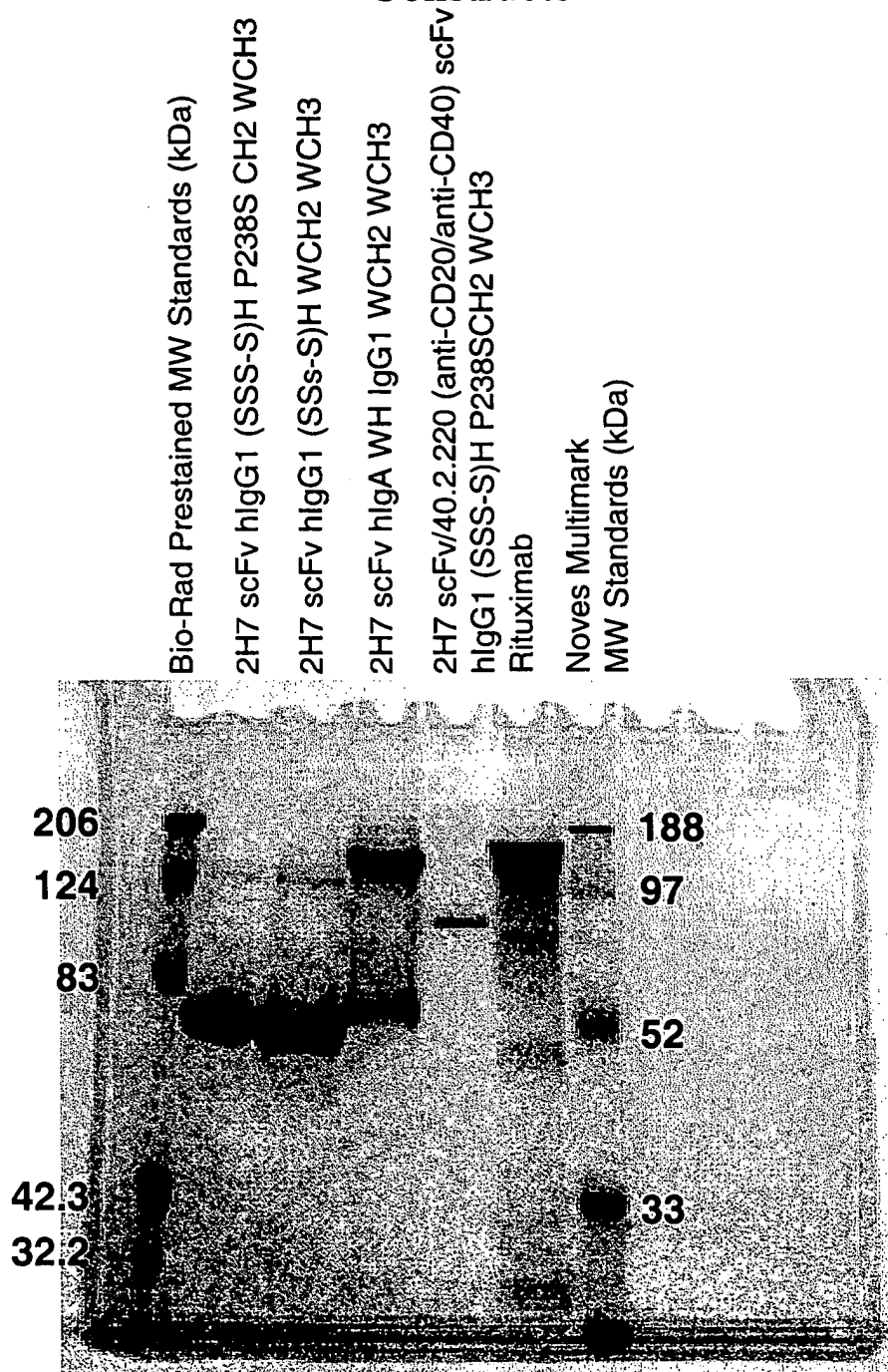
# Fig. 46

## ADCC mediated by 2H7 scFvIg Constructs by human PBMC effector cells against Bjab targets



Legend:
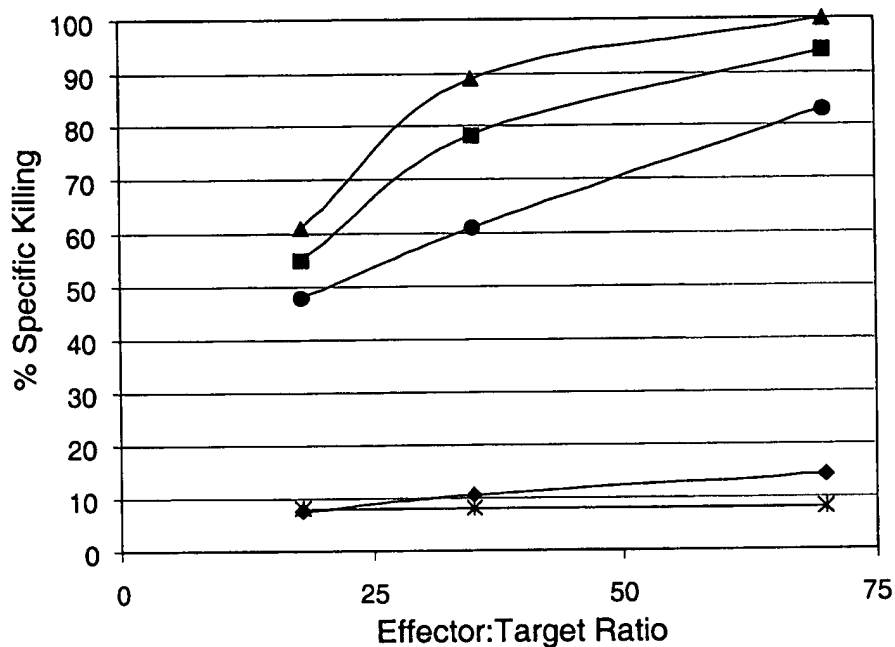
♦ 2H7 scFv hIgG1(SSS-S)H P238SCH2 WCH3   ● RITUXIMAB

▲ 2H7 scFv hIgA WH IgG1 WCH2 WCH3   ✱ CELLS ALONE (W/O AB)

■ 2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3

# Fig. 47

## Cell surface expression of anti-human CD3 G19-4 scFv hIgG1 (SSS-S)H P238SCH2 WCH3-hCD80TM/CT on Reh and T51 Cells.

### Reh anti-CD3 (G19-4) scFv hIgG1 (SSS-S)H P238SCH2 WCH3-hCD80TM/CT
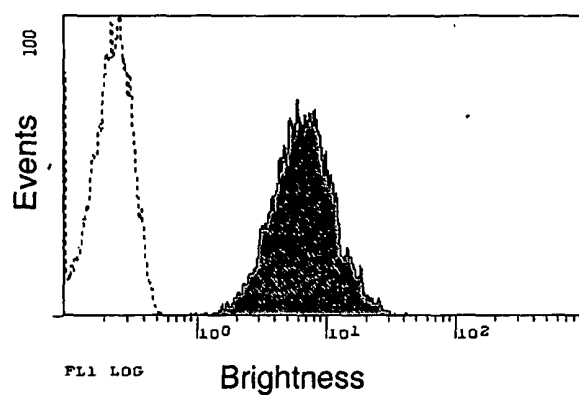


### T51 G19-4 scFv hIgG1 (SSS-S)H P238SCH2 WCH3-hCD80TM/CT:
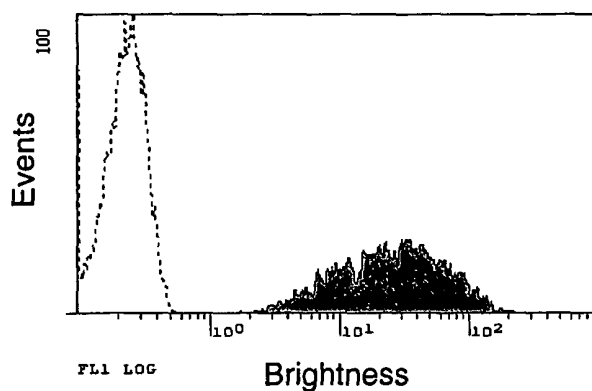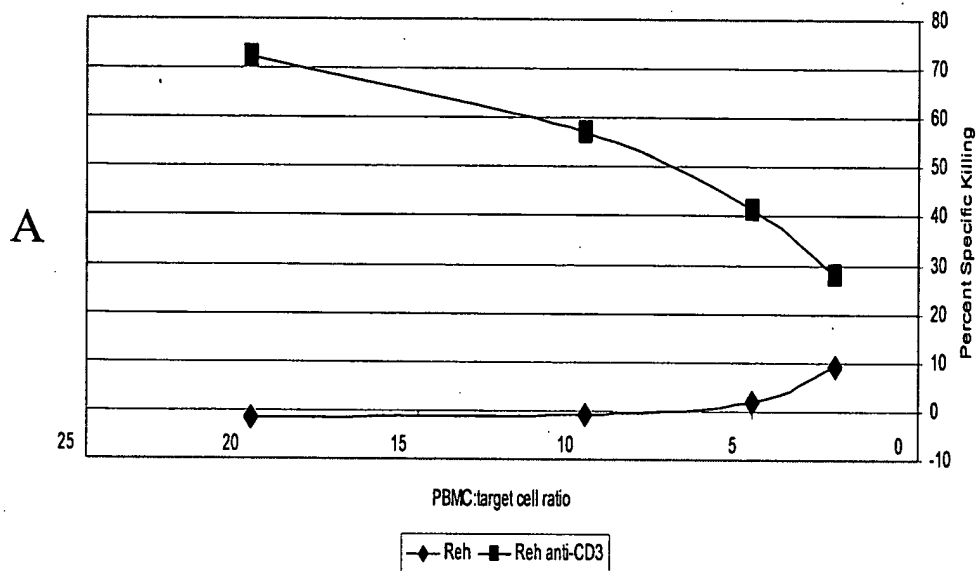
# Figure 48.
## Targeting of Cytotoxicity to Transfected Cell Lines by Surface expression of CD3 scFvIg

Cytotoxic activity of resting PBMC towards transfected Reh cells

A

PBMC:target cell ratio

Reh    Reh anti-CD3

Cytotoxic activity of resting PBMC towards transfected T51 lymphoblastoid cells

B

PBMC/target cell ratio

T51    T51 anti-CD3
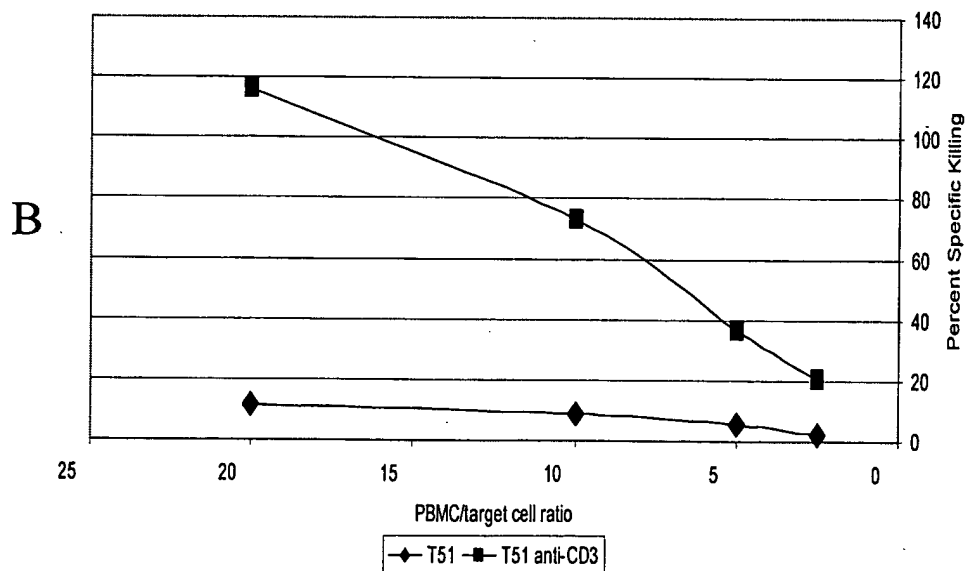
# Fig. 49

## Binding of 5B9, a mouse anti-human CD137 scFv hIgG1 (SSS-S)H WCH2WCH3 to stimulated human PBMC



Events

Brightness

Goat anti human IgG FITC

5B9 Hybridoma supernatant +
GAM IgG - FITC

5B9scFv hIgG1 (SSS-S)H WCH2 WCH3
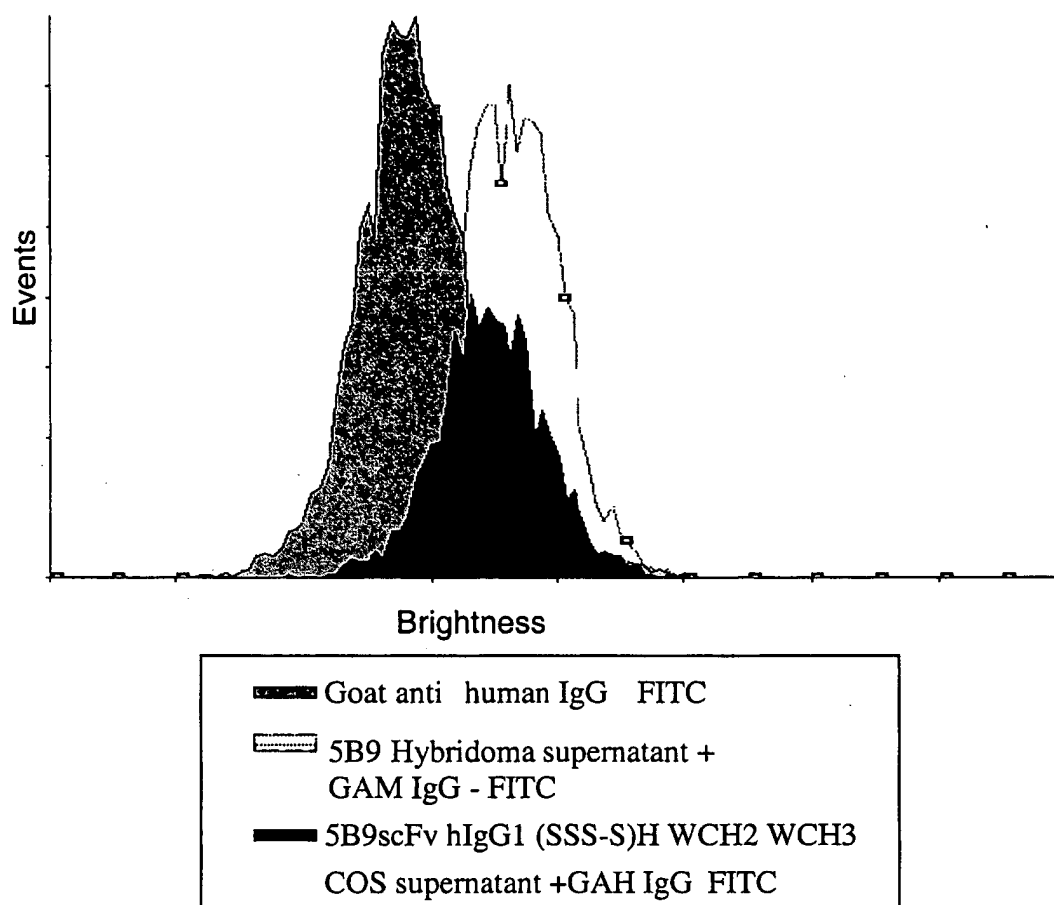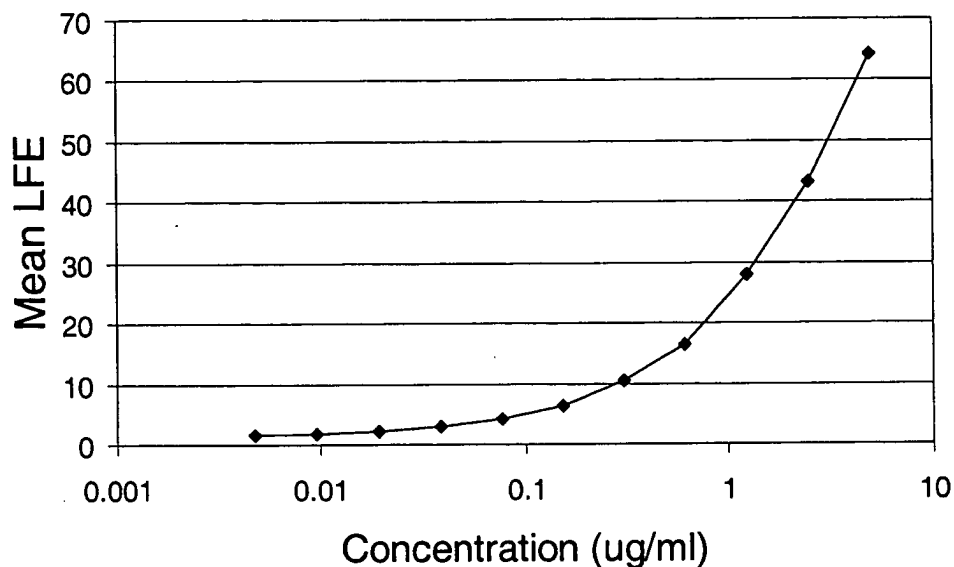COS supernatant +GAH IgG FITC

# Fig. 50
## Effect of $V_HL11S$ Mutation on CytoxB20
## 2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3 Protein Expression

### 50A. Standard Curve: 2H7VH-L11S-IgG1 (SSS-S)H WCH2 WCH3



### 50B. CHO supernatant Brightness and Estimation of Protein concentrations from Standard Curve:

| | CHO clone name | | | | |
| --- | --- | --- | --- | --- | --- |
| | **4F2** | **4F5** | **3E5** | **6B11A** | **2B8A** |
| Mean LFE | | | | | |
| 1/100 | 71.7 | 40.6 | 31.5 | 99.7 | 101.5 |
| 1/500 | 27.1 | 12.4 | 11.2 | 40.8 | 43 |
| **approx conc.** $\mu$g/ml | **600** | **225** | **125** | **1000** | **1250** |

# Fig. 51
## Production Levels of 2H7scFv VH L11S hIgG1
## (SSS-S)H WCH2 WCH3
## From CHO Clone Culture Supernatants
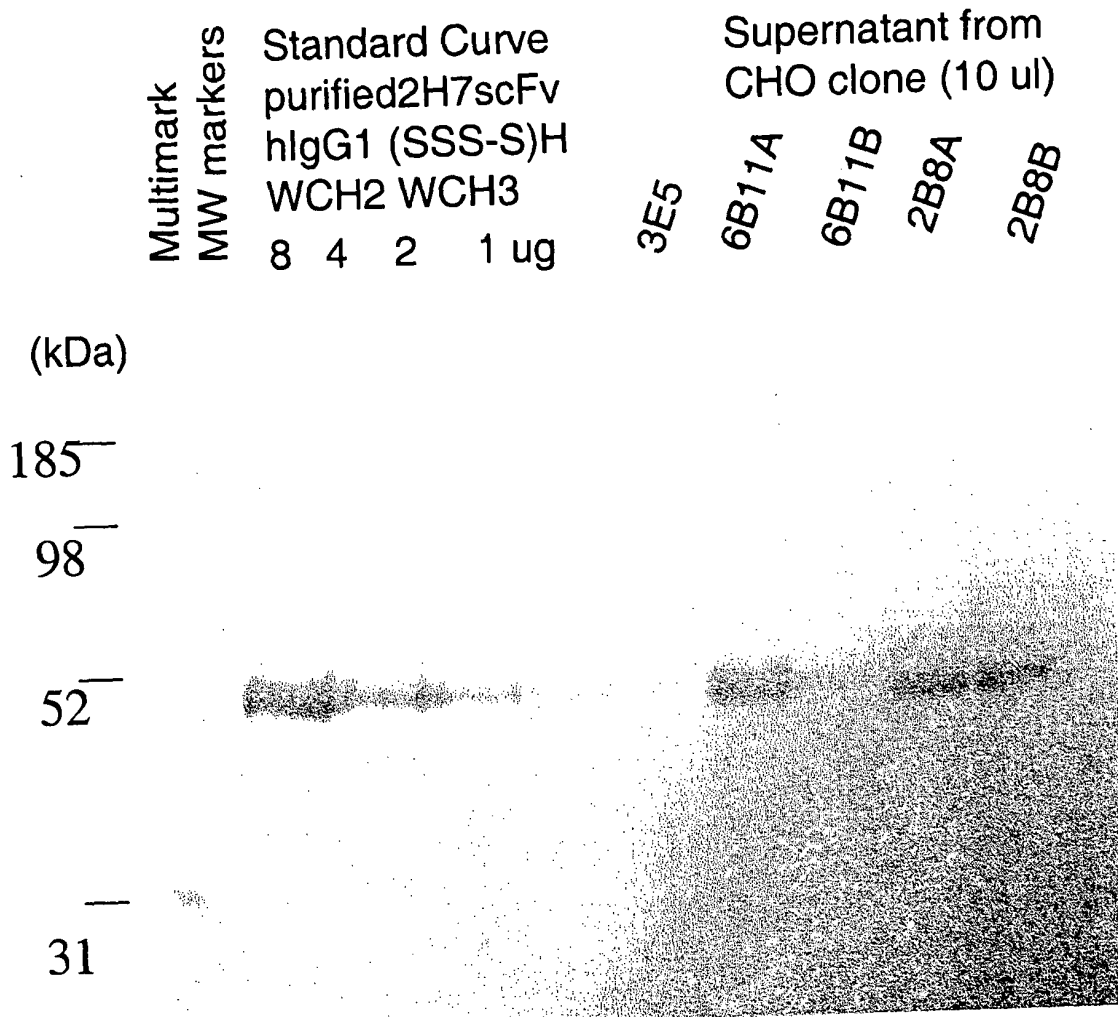
# Fig. 52
# Effect of VHL11S Mutation on G28-1 scFvIg Construct
# Protein Production from COS cells

### Binding of COS supernatants to Ramos cells



-◆- G28.1 scFv hIgG1(SSS-S)H WCH2 WCH3   -■- G28.1 scFv VHL11S hIgG1 (SSS-S)H WCH2 WCH3
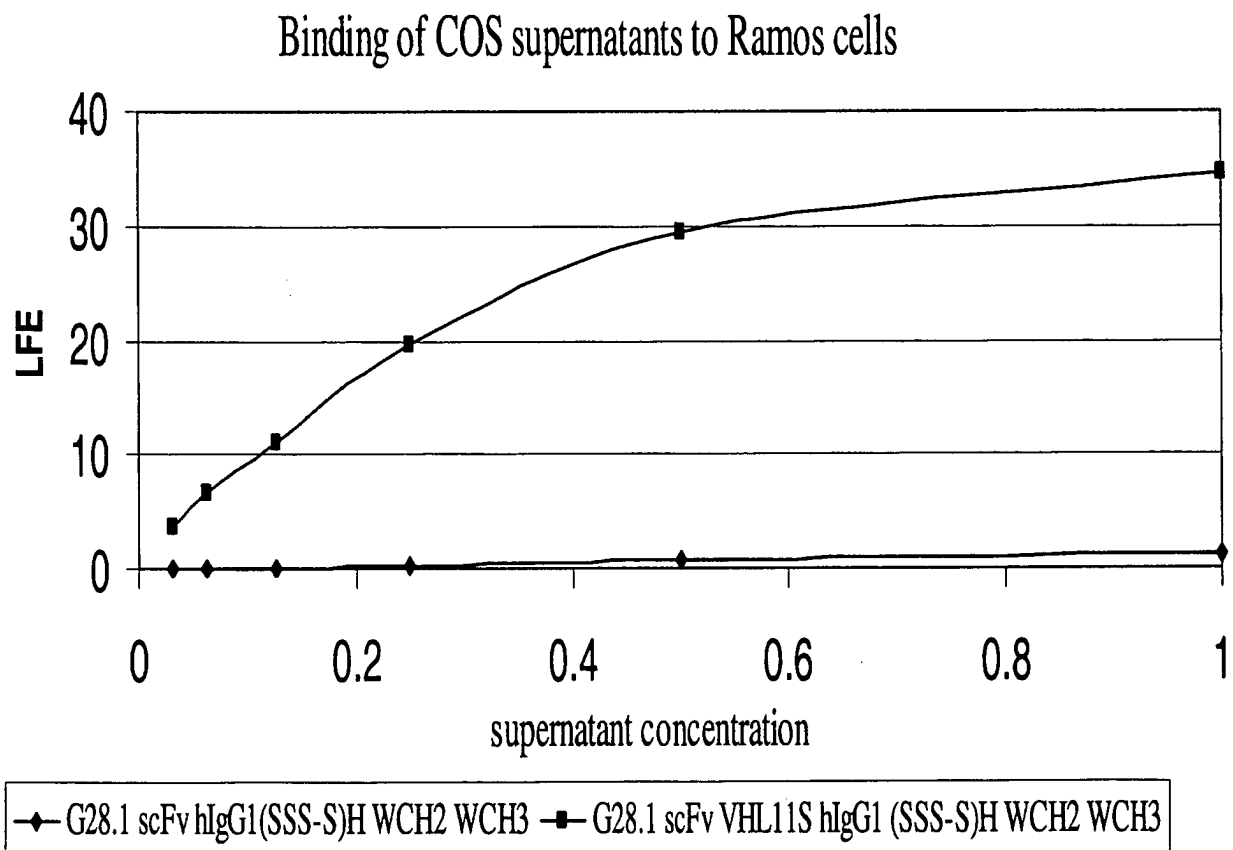
# Fig. 53

## Immunoblot of G28-1 scFvIg Constructs

Increased Protein Levels in COS supernatants
transfected with G28-1scFv hIgG1 (SSS-S)H WCH2 WCH3
After Substitution of Leucine with Serine at position 11 of VH (VHL11S)

### Fig. 53A.

Purified G28-1          G28-1 scFv
(11/6/01)              hIgG1 (SSS-S)H
scFv IgG1 (SSS-S)H      WCH2 WCH3
WCH2 WCH3              1 ul/well

80ng  40ng  20ng  10ng    A  B  C  D  E
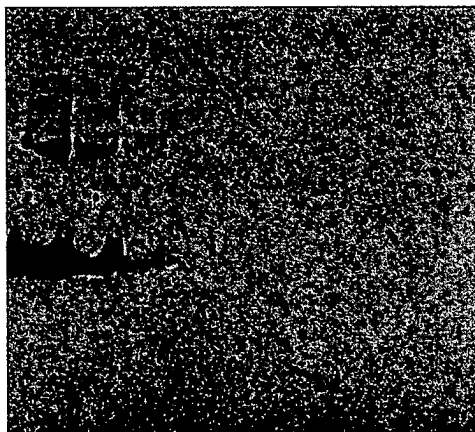
### Fig. 53B.

Purified G28-1          G28-1VHL11S
(11/6/01)            scFv hIgG1 (SSS-S)H
scFv hIgG1(SSS-S)H      WCH2 WCH3
WCH2 WCH3              1 ul/well
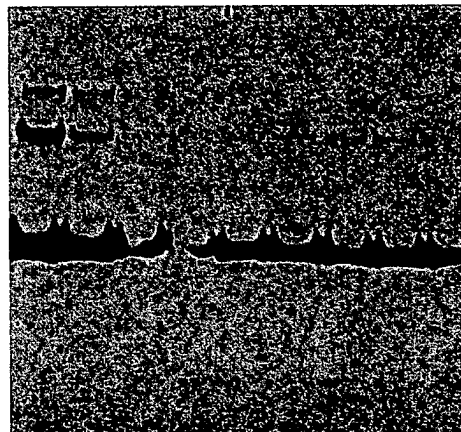
80ng  40ng  20ng  10ng    A  B  C  D  E

# Fig. 54

## Binding of 2H7 scFvIg Constructs with Altered Hinges and CH3 domains to CD20 CHO Cells
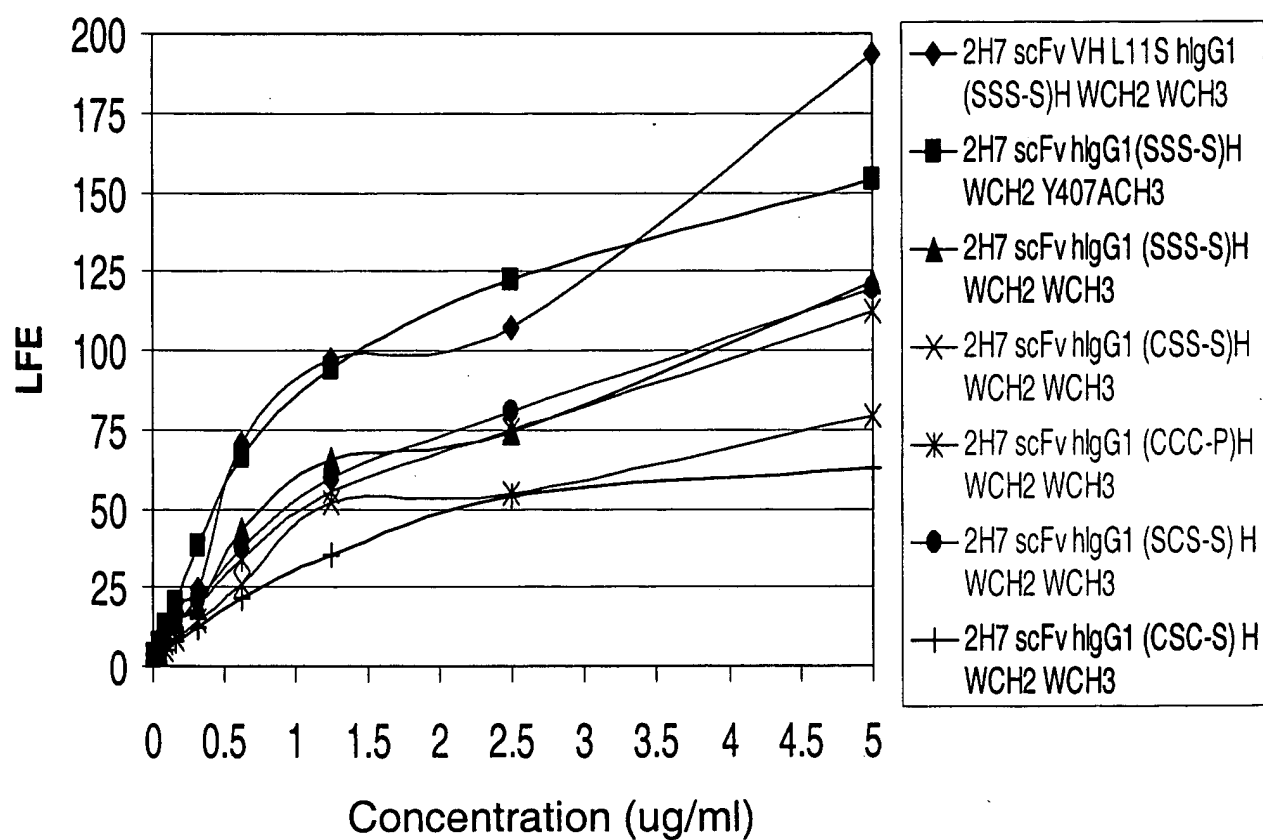
# Fig. 55

## ADCC Activity of 2H7 scFvIg constructs Against
## BJAB Targets and PBMC Effectors



Legend:
- 2H7 scFv hIgG1 (CCC-P)H WCH2 WCH3
- 2H7 scFv hIgG1 (CSS-S)H WCH2 WCH3
- 2H7 scFv hIgG1 (SCS-S)H WCH2 WCH3
- 2H7 scFv hIgG1 (CSC-S)H WCH2 WCH3
- 2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3
- 2H7 scFv hIgG1 (SSS-S)H WCH2 Y407ACH3
- 2H7 scFv hIgA WH WCH2 WCH3
- Natural Killing

X-axis: Effector:Target Ratio
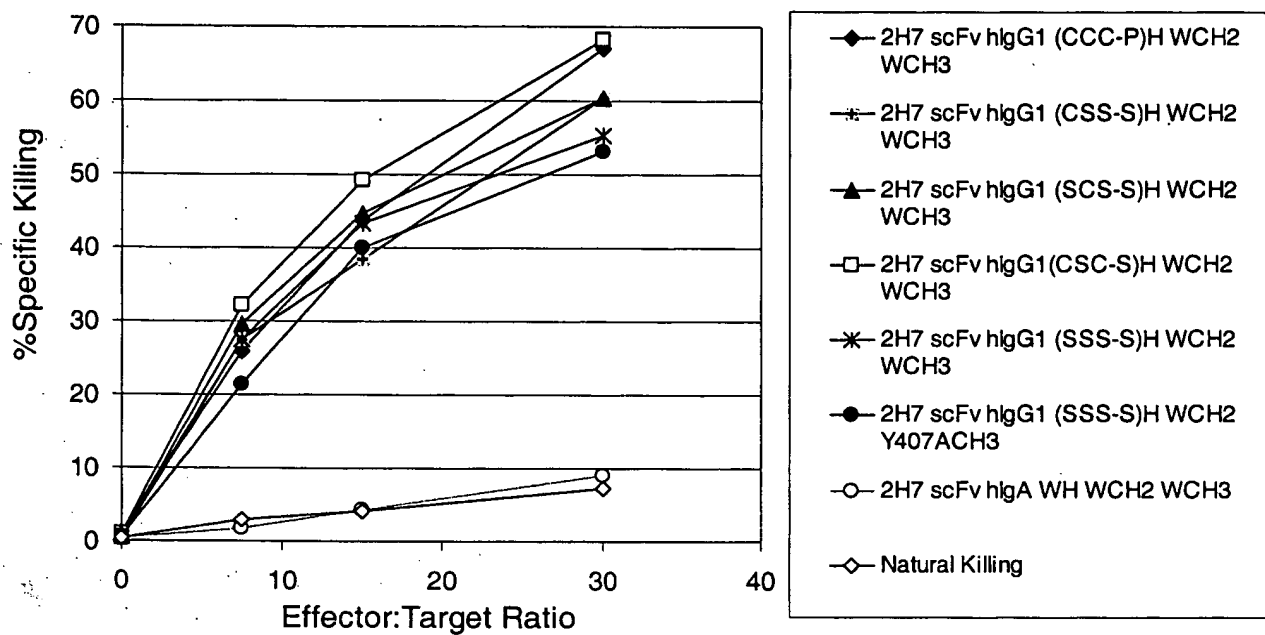Y-axis: %Specific Killing

# Fig. 56

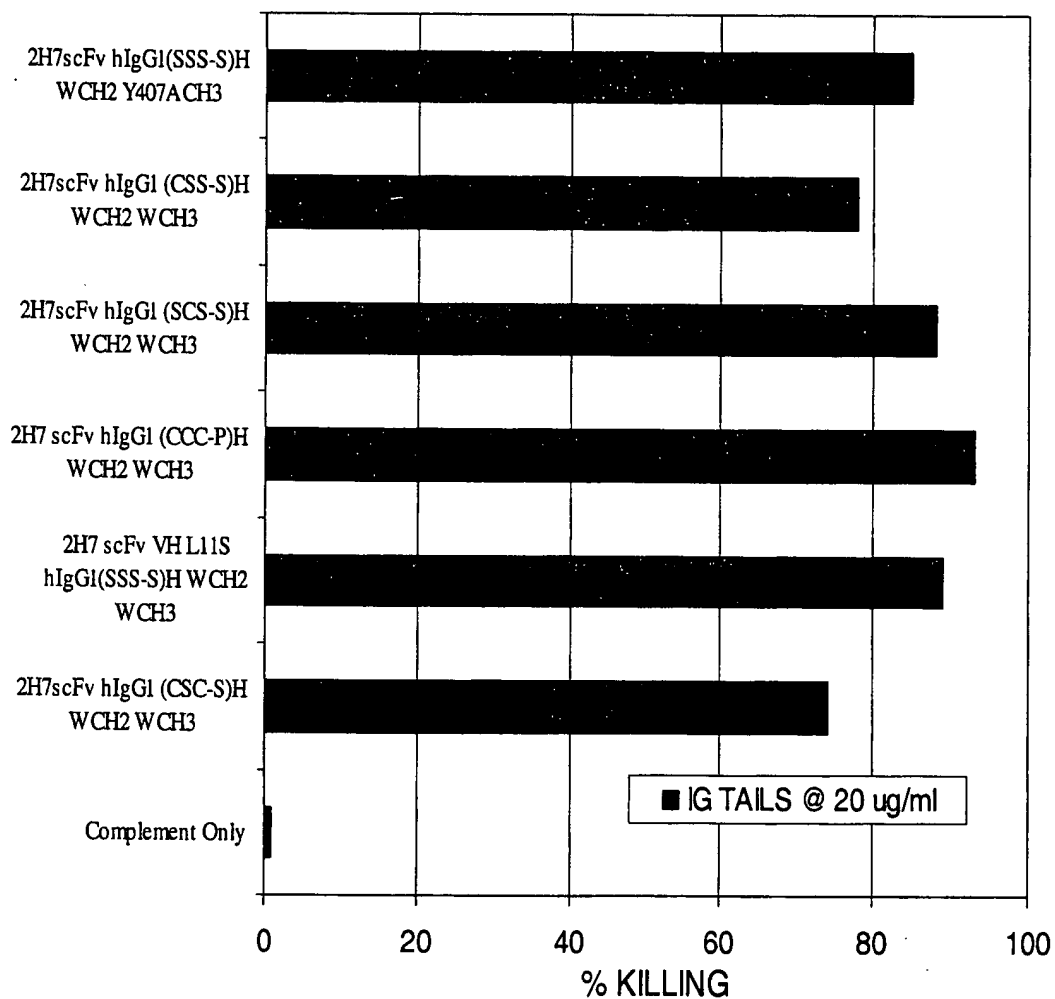## Complement Activity of 2H7 scFvIg Constructs
## With Ramos Target Cells

# Fig. 57

## Binding of 2H7 scFvIg Derivatives to CD20CHO Cells

A. ■ No fusion protein

B. ■ 2H7 scFv hIgE CH2CH3CH4

C. ▦ 2H7 scFv hIgA WH WCH2 WCH3

D. ■ 2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3

### FITC anti-hIgG
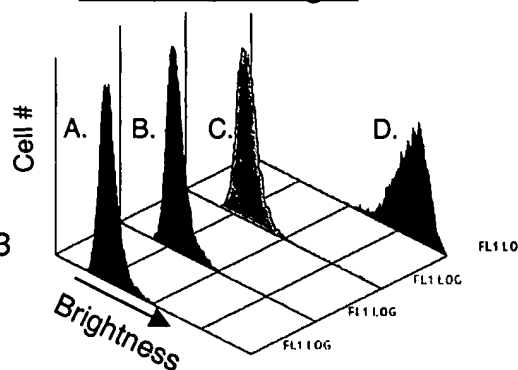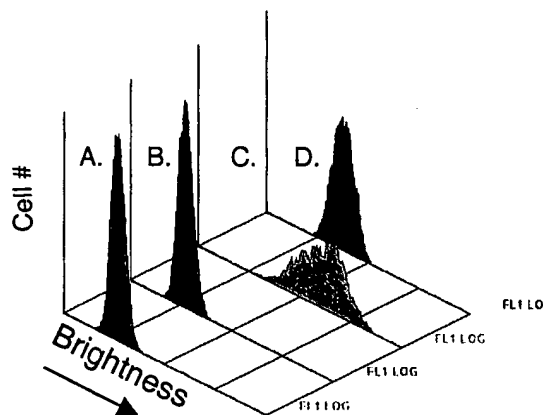
### FITC anti-hIgA

### FITC anti-hIgE
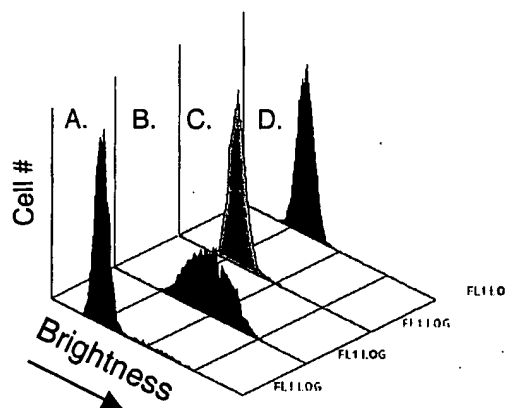
# Fig. 58

Fig. 58A.  2H7 scFv VH L11S human IgE (WCH2 WCH3 WCH4)
Binding to CD20 CHO at 30 ug/ml
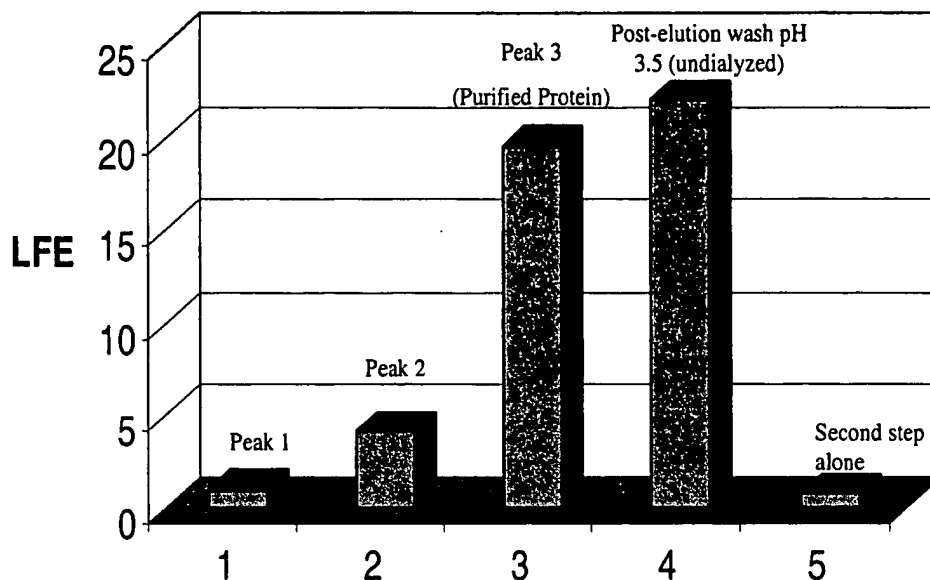


Fig. 58B.  ADCC Activity of 2H7 VHL11S IgE (WCH2  WCH3 WCH4)
Protein Fractions with **PBMC** Effectors and Bjab Targets
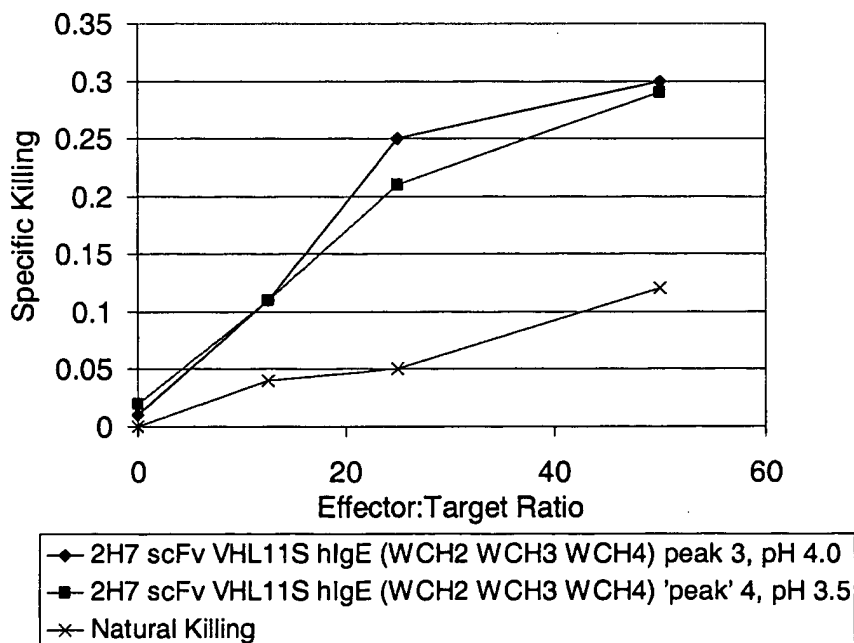
# Fig. 59

Binding Data for COS derived α-CD20 (2H7) scFv VHL11S
mIg E (WCH2 WCH3 WCH4) and
mIgA (WH WCH2 WCH3)Tailed Molecules



Binding of 2H7 VHL11S murine constructs to CD20 CHO cells

# Fig. 60
## HPLC Profiles of 2H7 scFvIg Constructs



**A**

2H7 scFv hIgG1
(SSS-S)H P238SCH2 WCH3

**B**

2H7 scFv hIgG1
(CSS-S)H WCH2 WCH3

**C**

2H7 scFv hIgG1
(SCS-S)H WCH2 WCH3

**D**

2H7 scFv hIgG1
(SSS-S)H WCH2 (Y407A)CH3

Injection
Point ($t_o$)

Time After Injection

## Fig. 61
### HPLC Profiles of 2H7 scFvIg Constructs

Size Standards:  670   158   44   17   1.35

**A**

2H7 scFv hIgG1
(SSS-S)H WCH2 WCH3

100    75

300

**B**

2H7 scFv hIgG1
(CSC-S)H WCH2 WCH3

75

**C**

2H7 scFv hIgG1
(CCC-P)H WCH2 WCH3

100

**D**

2H7 scFv hIgA WH
hIgG1WCH2 WCH3

150

Injection
Point (t$_o$)

Time after Injection

## Fig. 62

HPLC Profiles of 2H7 scFvIg Constructs:

Size Standards:   670   158   44   17   1.35

**A**
2H7 scFv hIgG1
(SSS-S)H WCH2 WCH3

Injection
Point (t$_0$)                    Time after Injection

670   158  44        17    1.35

**B**
2H7 scFv hIgG1
(SCS-S)H WCH2 WCH3                                75

**C**
2H7 scFv hIgA
WH WCH2 T4CH3

**D**
2H7 scFv hIgG1 (SSS-S)H
WCH2(F405A Y407A) CH3

Time after Injection

# Fig. 63

**Binding of Purified Proteins from COS Supernatants
to CD20 CHO cells:
Differential Effects of CH3 Mutations on Binding**



—◆— 2H7 scFv VHL11S hIgG1 (SSS-S)H WCH2 WCH3
—■— 2H7scFv VHL11S hIgG1 (SSS-S)H WCH2 (F405A Y407A) CH3

# Fig. 64

Binding of FITC conjugated 2H7 scFv VHL11S hIg Proteins to
CD20 CH0 Cells



Legend:
- —▲— 2H7 scFv VH L11S hIgG1 (CSC-S)H WCH2 WCH3
- —✕— 2H7 scFv VH L11S hIgG1(CSS-S)H WCH2 WCH3

# Fig. 65

Nonreducing SDS-PAGE on Protein A-Purified Lots
of 2H7 scFv VHL11S hIg Constructs (10 ug/lane)
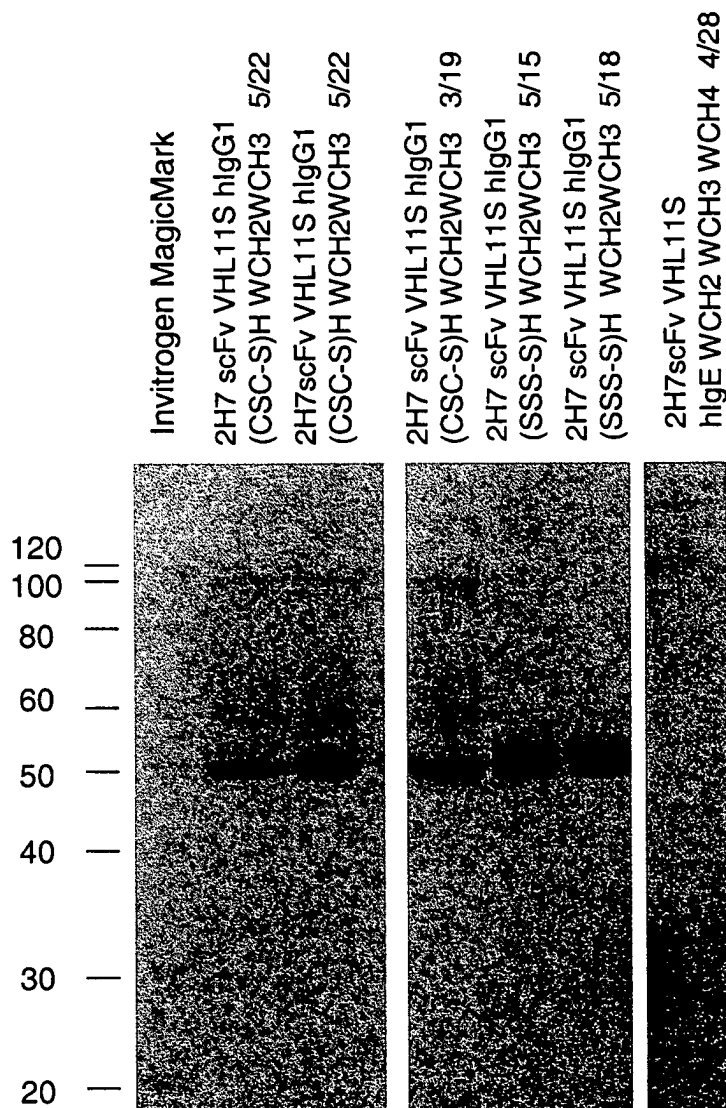
# Fig. 66

## Alterations in Human IgG Fc sequence
## that differentially change effector function efficiency

P238S   240   250   T256N   260   270   280   K290Q   300

```
hIgG2   KCCVECPPCPAPPVAG-PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGMEVHNAKTKPREEQFNSTF
hIgG3   DTPPPCPRCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFKWYVDGVEVHNAKTKPREEQYNSTF
hIgG4   KYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTF
hIgG1   DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTF
rIgG2b  PTCPTCHKCPVPELLGGPSVFIFPPKPKDILMISCNAKVTCVVVDVSEEEPDVCFSWFVNNVEVHTACTCPREECYNSTF
```
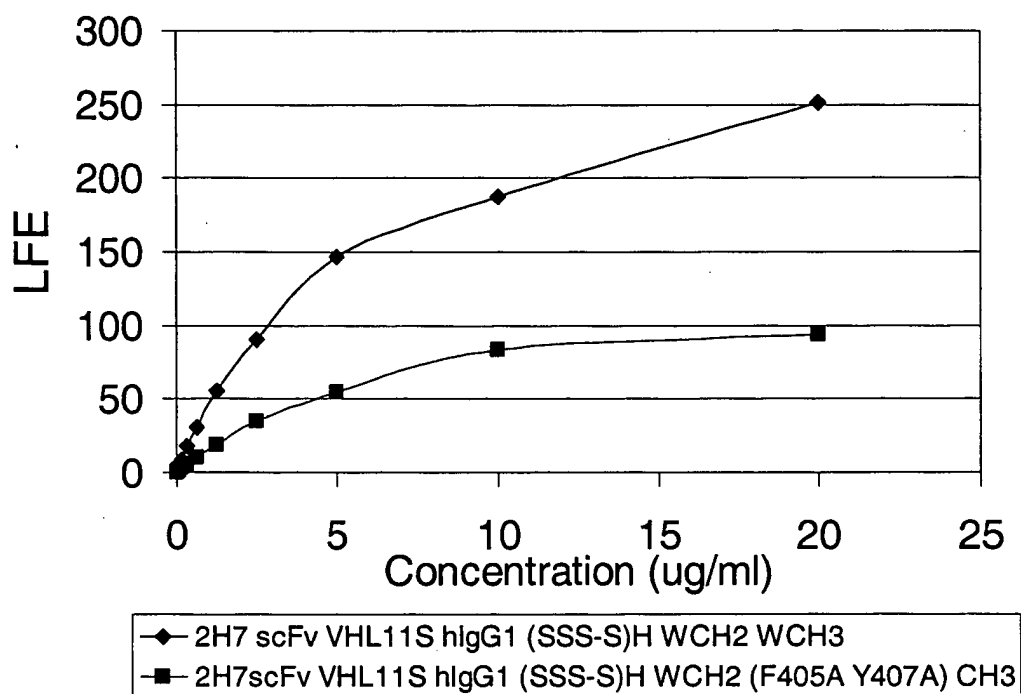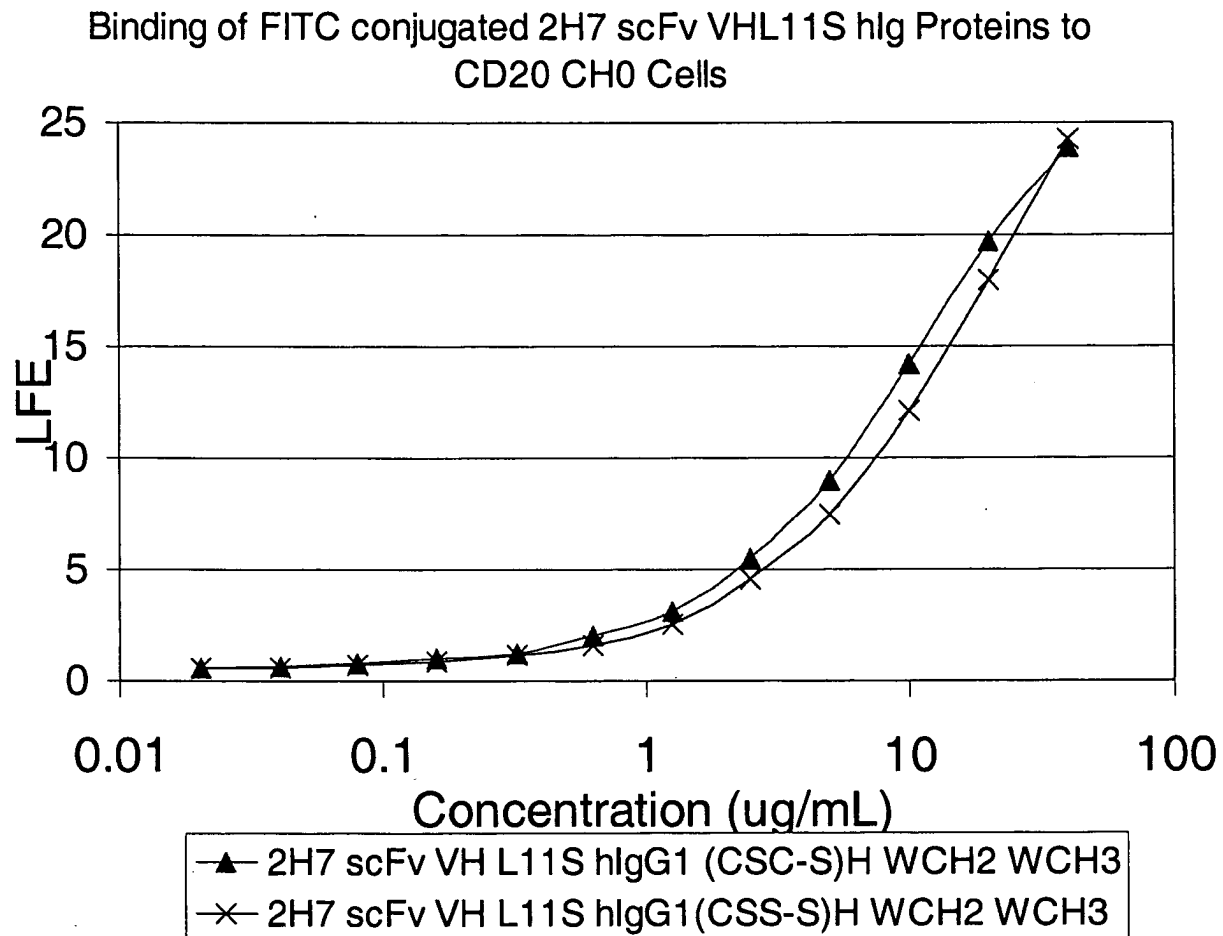
300   310   320   K322S   330   P331S   340   A339P

```
hIgG2   IFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV
hIgG3   IFRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAM
hIgG4   IYRVVSVLTVLHQDWLXGKEYKCKVSXKGLPSSIEKTISXAXGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV
hIgG1   IYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEMTKNQVSLTCLVKGFYPSDIAV
rIgG2b  IFRVVSALPICHCDWMSGKEFKCKVNNKALPSFIEKTISKFKGLVRKPCVYVAGPPTEQILTEGTVSLTCLTSGFLPNDIGV
```

■ CDC residue    ■ ADCC

# Figure 67.

ADCC Activity of 2H7 scFv VHL11S hIgG1 (CSC-S)H WCH2 WCH3 from
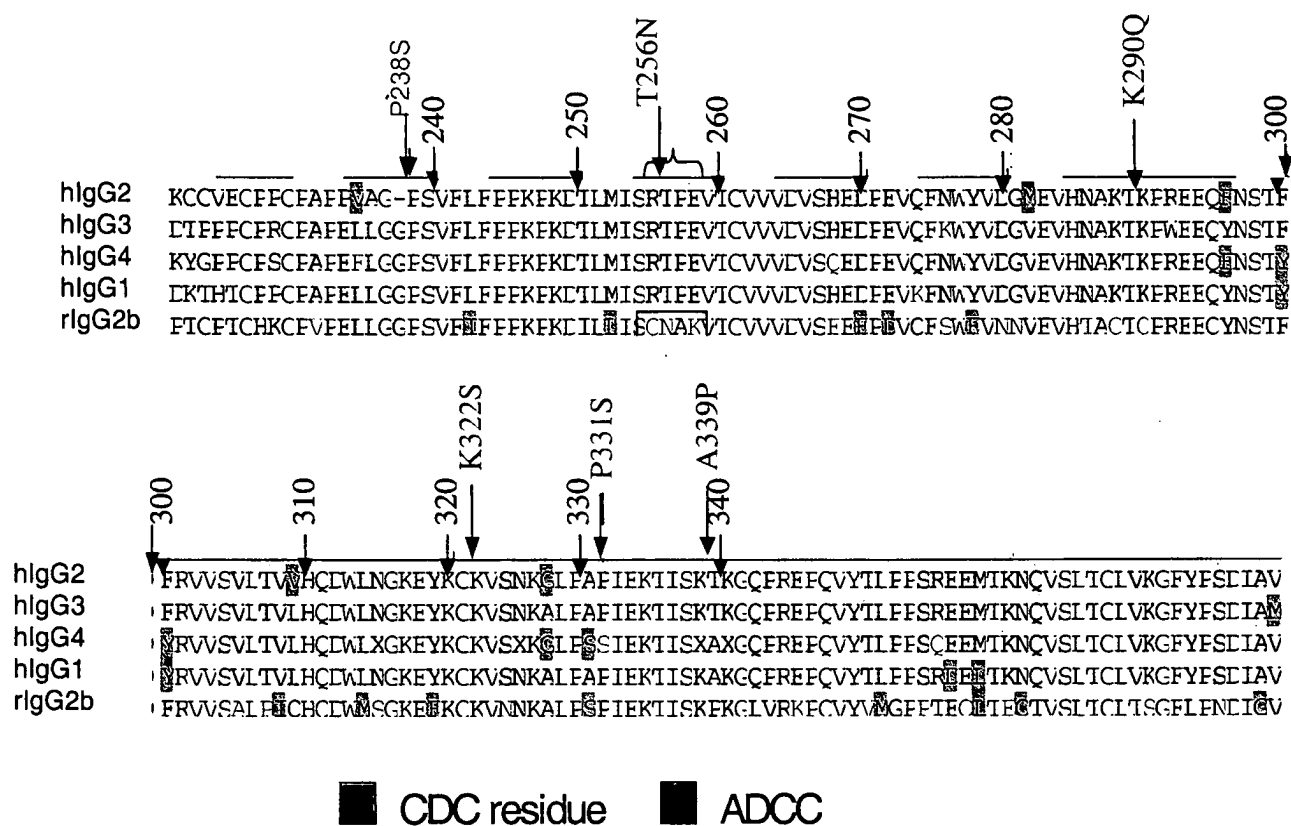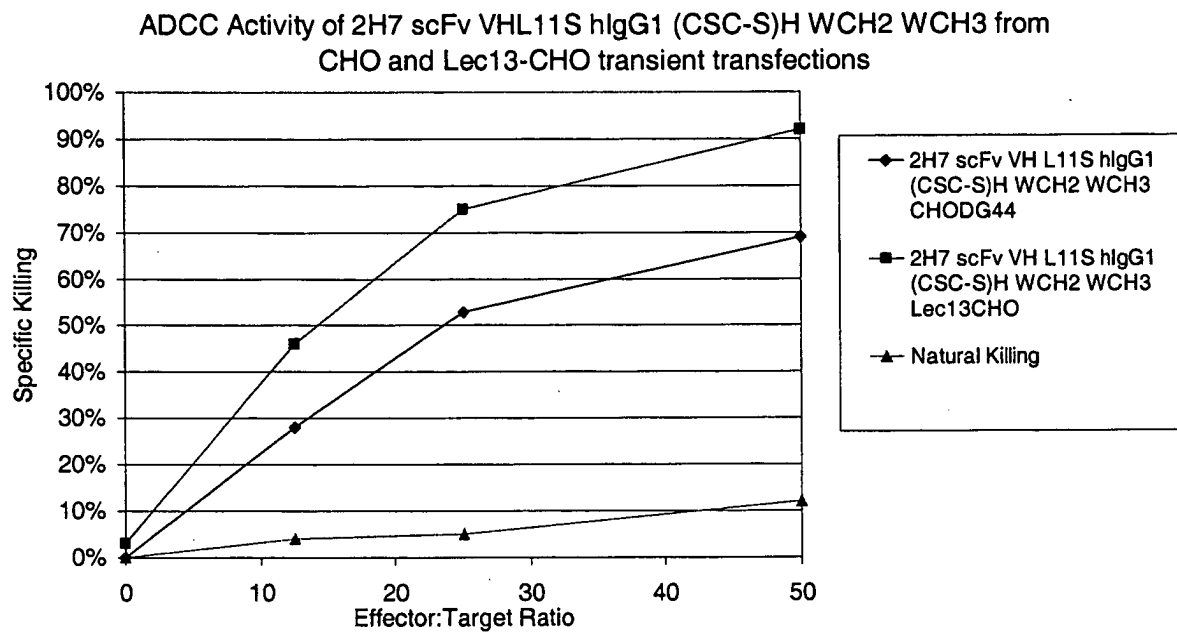CHO and Lec13-CHO transient transfections

# Fig. 68

## CD16(ED) hIgG1(SSS-S)H P238S CH2 WCH3 high and low affinity alleles expressed as soluble molecules

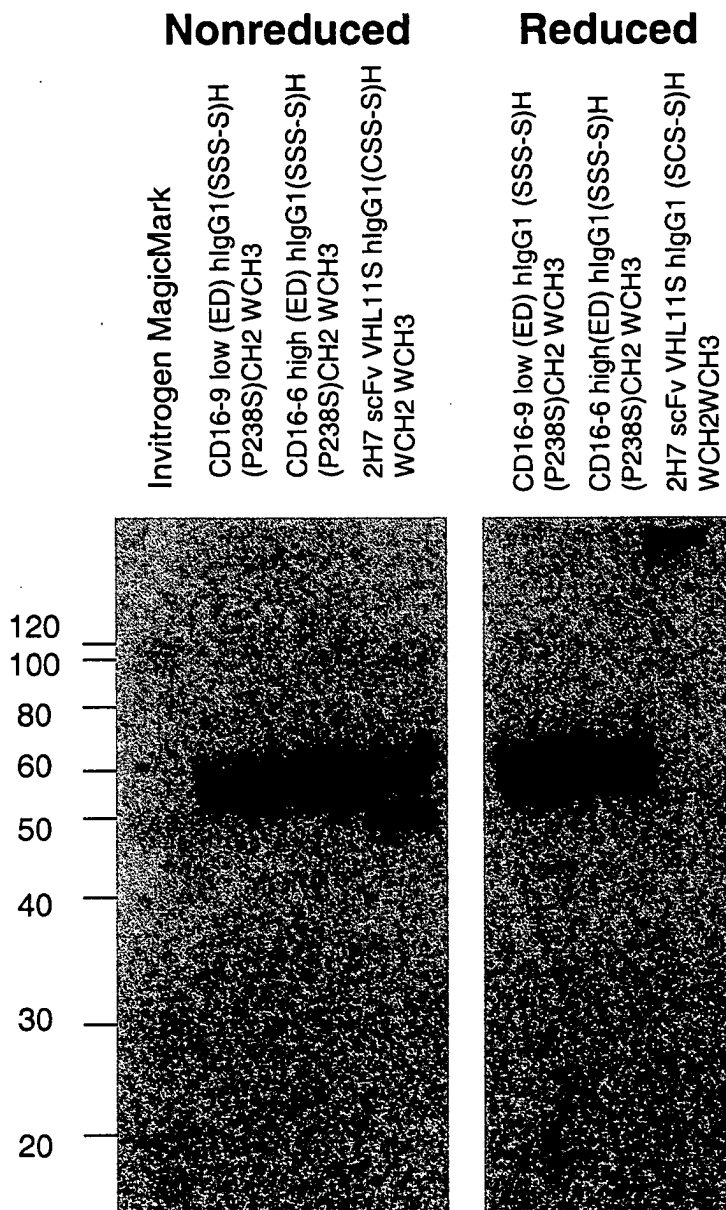# Fig. 69

Binding of soluble CD16-FITC high and low affinity fusion proteins
to 2H7 scFv VHL11S hIgG1 (CSC-S)H WCH2WCH3 or
(SSS-S)H (P238S)CH2WCH3 on CD20CHO Targets



Legend:
- → 2H7 scFv VH L11S hIgG1 (CSC-S)HWCH2WCH3 + (high)
- ■ 2H7 scFv VH L11S hIgG1 (SSS-S)H P238SCH2 WCH3 + (high)
- ▲ 2H7 scFv VH L11S hIgG1 (CSC-S)H WCH2WCH3 + (low)
- ✳ 2H7 scFv VH L11S hIgG1 (SSS-S)H P238SCH2 WCH3 + (low)

# Fig. 70

Binding of FITC Labeled, Recombinant Human
CD16(ED) extracellular domain -Ig Fusion Protein to
CytoxB Derivatives on CD20 CHO Cells



FITC-CD16 ED(158 V/F)
hIgG1(SSS-S)H (P238S)CH2 WCH3

CD20+

CytoxB20
2H7 scFv hIgG1

Expression of surface displayed SMIPs links
modified cDNAs with the altered fusion proteins

scFvIg1

scFvIg2

Mammalian
Cell Transfected
With
1.A single surface displayed
scFvIg expression construct
OR
2.a library of such molecules

scFvIg1

# Fig. 71
## CD37 mAbs and scFvIg Induce Apoptosis

| | Bjab Staining | Annexin V Positive | |
|---|---|---|---|
| | No scFvIg | 17.5 | |
| | 2H7 MH | 27 | |
| | G28-1 MH | 30.6 | |
| scFvIg | G28-1 IgAH | 28.9 | |
| | HD37 MH | 29.1 | |
| | (2H7+G28-1)MH | 41 | |
| | (2H7+HD37) MH | 37.1 | |
| | (G28-1+HD37) MH | 35.3 | |
| | | | |
| | | | |
| | | | plus GAM |
| | Ramos | AnnexinV Positive | AnnexinV positive |
| | cells alone | 3 | 3.3 |
| | 2H7 Mab | 1.4 | 3.1 |
| | G28-1 Mab | 18.3 | 8.7 |
| mAbs | HD37 Mab | 3.7 | 3.1 |
| | G28-5 | 3.9 | 8.3 |
| | 2H7+G28-1 | 32.3 | 35.7 |
| | 2H7+HD37 | 5 | 10.5 |
| | 2H7+G28-5 | 5.7 | 19.4 |
| | HD37+G28-1 | 26.9 | 50 |
| | HD37+G28-5 | 8.2 | 18.4 |
| | G28-1+G28-5 | 39.5 | 68.3 |
| | | | |